POLYMORPHISM OF THE GLUTATHIONE S-TRANSFERASE M1 AND CYTOCHROME P4501A1 GENES AND SUSCEPTIBILITY TO EMPHYSEMA AND LUNG CANCER

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DECLARATION

I declare that the experiments outlined here were carried out by myself, unless otherwise stated, between November 1992 and July 1995. This thesis was composed by myself.

Ann M Cantlay 20 June 1997

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ABSTRACT

Emphysema is a lung disease characterised by abnormally large airspaces in the lungs resulting from airspace wall destruction. In centriacinar emphysema the airspace enlargement involves the respiratory bronchioles, while in panacinar emphysema airspace enlargement is uniform across the alveoli and respiratory bronchioles. These 2 patterns of emphysema are thought to result from different pathological mechanisms. Glutathione S-transferase M1 (GSTM1) detoxifies components of cigarette smoke, but polymorphic gene deletion occurs in approximately 50% of populations. Cytochrome P4501A1 (CYP1A1) mediates the metabolic activation of compounds in cigarette smoke. A genetic polymorphism of CYP1A1 is thought to increase the efficiency of the enzyme, increasing the amount of reactive species generated through metabolism. Since the GSTM1 and CYP1A1 gene polymorphisms have been associated with lung cancer and both enzymes metabolise components of cigarette smoke they represent ideal candidates for investigation into genetic susceptibility to emphysema. Expression of GSTM1 in lung tissue by immunohistochemistry could not be differentiated from other GSTM enzymes, including the highly homologous enzyme GSTM4. Reverse transcription PCR was therefore used to identify expression of GSTM1, where there was an intact gene, and GSTM4 in lung tissue. Deletion of GSTM1 was associated with emphysema in the presence of concomitant lung cancer, but not with lung cancer alone. No association between the GSTM1 polymorphism and either centriacinar or

panacinar patterns of emphysema could be demonstrated, but a small association was found in cases with both centriacinar and panacinar emphysema. The glutathione S-transferase M1 deletion was not associated with chronic obstructive pulmonary disease, the clinical manifestation of emphysema, nor with the severe emphysema found in autopsy specimens. The cytochrome P4501A1 polymorphism was associated with emphysema concomitant with lung cancer, but no association could be demonstrated with a specific pattern of disease. Furthermore, the CYP1A1 polymorphism was found to be associated with very early emphysema, detectable only by microscope. No association was found between cytochrome P4501A1 and lung cancer, nor with chronic obstructive pulmonary disease. This study demonstrates that the GSTM1 and CYP1A1 polymorphisms have a small, but significant, association with emphysema, which cannot be explained by concomitant lung cancer, since lung cancer alone was not associated with either polymorphism. The association of the polymorphisms with mild emphysema, in particular that seen between CYP1A1 and microscopic emphysema, and not with the severe emphysema represented by chronic obstructive pulmonary disease, and in the case of GSTM1 with autopsy emphysemas, indicates that these enzymes have a general role in the early protection of the lung against damage caused by xenobiotics such as cigarette smoke. Sequencing of the CYP1A1 gene identified a novel polymorphism, a C to A substitution, which results in an amino acid change from threonine to asparagine at positon 461of the gene. This amino acid is adjacent to the previously characterised exon 7 isoleucine to valine polymorphism, but is nearer to the cysteine residue thought to be of importance for haem binding of the enzyme. Of 26 Scottish

individuals previously genotyped as heterozygotes for the Ile-Val^{462} polymorphism, 14 were subsequently shown to actually have the Thr-Asn^{461} polymorphism. These preliminary data indicate that in the Scottish population there is a polymorphism which may be more common than the Ile-Val^{462} polymorphism. Further studies will identify whether or not this novel polymorphism has a functional effect on the enzyme and hence on disease susceptibility.

ABBREVIATIONS

GST	Glutathione S-transferase gene or enzyme, for example GSTM1 refers to glutathione S-transferase M1
CYP	Cytochrome P450 gene, for example CYP1A1 refers to cytochrome P4501A1
P450	Cytochrome P450 enzyme
COPD	Chronic obstructive pulmonary disease
FEV1	Forced expiratory volume per second
CT	Computed tomography
RB	Respiratory bronchiole
AS	Alveolar sac
AD	Alveolar duct
TB	Terminal bronchiole
Lm	Mean linear intercept
AWUV	Alveolar wall surface area per unit volume of lung tissue
α1Pi	Alpha 1-antiprotease
BAL	Bronchoalveolar lavage
GSH	Glutathione
DNA	Deoxyribonucleic acid
SOD	Superoxide dismutase
H_2O_2	Hydrogen peroxide
mRNA	Messenger RNA
RNA	Ribonucleic acid
RFLP	Restriction fragment length polymorphism
SSCP	Single strand conformational polymorphism
GIT	Gastrointestinal tract
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
EROD	Ethoxyresorufin-O-deethylase
XRE	xenobiotic responsive element
BTE	basic transcription element
Ah	aryl hydrocarbon
AHH	Aryl hydrocarbon hydroxylase
PAH	Polycyclic aromatic hydrocarbon
RT-PCR	Reverse transcription polymerase chain reaction
BS	Tris buffered saline
PCR	Polymerase chain reaction
$MgCl_2$	Magnesium chloride
cDNA	Complementary DNA
X-Gal	5-bromo-4chloro-3-inolyl-β-D-galactoside
IPTG	β-D-isopropyl-thiogalactopyranoside
EDTA	Ethylenediaminetetra acetic acid
FT	Fisher's exact test
Ile462	Allele of the CYP1A1 gene coding for an isoleucine residue at amino acid position 462

Val462	Allele of the CYP1A1 gene coding for an valine residue at amino acid position 462
Thr461	Allele of the CYP1A1 gene coding for an threonine residue at amino acid position 461
Asn461	Allele of the CYP1A1 gene coding for an asparagine residue at amino acid position 461

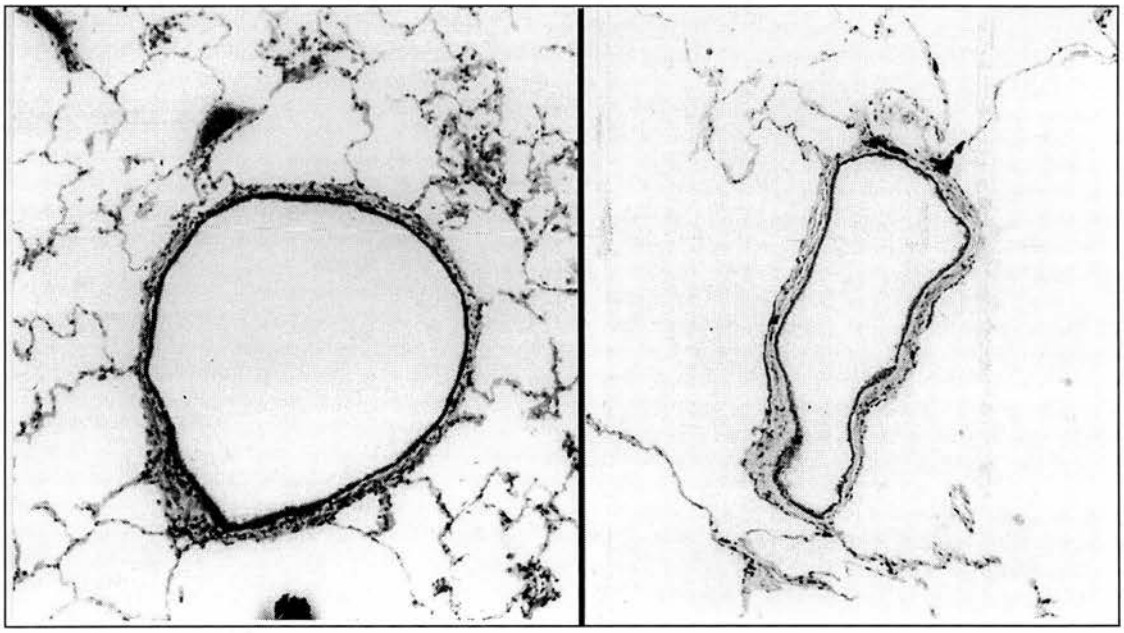
1.1 Emphysema

Emphysema is a common lung disease which is characterized by loss of alveolar tissue leading to increased airspace size. It is present, with variable severity, in one third of all adult males, and at least half of male smokers (Pratt, 1988). Although emphysema is related to cigarette smoke exposure, this is by no means exclusive, and approximately 11% of non smokers will have emphysema, although severity is generally much less compared to the severity found in the lungs of smokers (Pratt, 1988).

1.1.1 Chronic obstructive pulmonary disease

Clinically, emphysema presents as chronic obstructive pulmonary disease. Emphysema, by definition, involves destruction of airspace walls. The walls of the airspaces are attached to each other and to the small airways which supply them. This attachment of the airspace walls allows these airways to maintain a circular circumference by elastic pull from the walls. If the airspace wall attachments are destroyed, the bronchiole circumference cannot be maintained, the airway collapses inwards, and the volume of air which can pass through the airway is reduced (Lamb, 1995), Figure 1.1. Chronic obstructive pulmonary disease is characterized by a fixed airways resistance (Pride, 1995). Asthma also presents as increased airflow resistance, which is characteristically reversible, and is therefore not generally included under the

term COPD (Pride, 1995). This term refers to lung disease which results in reduced lung function, as measured by the forced expiratory flow of the individual in one second (FEV1). Symptoms of chronic obstructive pulmonary disease include persistent cough, purulent sputum, and breathing difficulty, particularly during exercise. The damage caused to the lungs by emphysema is irreversible (Lamb, 1995).



A B

Figure 1.1 Photomicrographs of the cross-section of two small airways. Magnification X50. (A) shows a bronchiole from a non-smoking individual which shows no sign of disease. The alveolar walls surrounding the bronchiole provide support which maintains the airway in a circular circumference. (B) shows an airway from a patient with mild panacinar emphysema. The reduction in alveolar walls and their attachments to the bronchiole has resulted in a collapsed, eliptical circumference.

Published figures on COPD mortality are probably underestimates (Strachan, 1995), since reduced levels of FEV1 have been shown to be risk factors for other diseases, including coronary heart disease and stroke. Emphysema and COPD are not only important diseases for study because of their high mortality rates, they also account for a large proportion of sickness leave and are a heavy burden on the health services (Strachan, 1995).

1.1.2 Morphology of emphysema

Emphysema is in essence a morphologically defined disease with a constellation of recoginsed but nonspecific clinical symptons and signs. Therefore, while diagnosis can be made using CT scanning (MacNee *et al*, 1991), accurate and precise determination of pattern of disease and severity requires direct morphological examination of lung tissue.

Emphysema is defined as "enlargement beyond normal of the airspaces of the lung, accompanied by airspace wall destruction, with no obvious fibrosis" (Snider *et al*, 1985). Within the generic term of emphysema, four main patterns of lung damage are generally recognized. These patterns are centriacinar, panacinar, paracicatricial and paraseptal. It is believed that these different patterns may reflect as yet unknown different pathological causes (Kim *et al*, 1995).

The normal architecture of the lung is comprised of a series of branching airways, which become progressively smaller, before ending in sacs called alveoli. From the mouth or nose, air travels through the trachea, which divides to form two bronchi. These bronchi undergo further sub divisions to create the bronchioles which, following division, become respiratory airways. The respiratory bronchioles lead into the alveolar ducts, and these final structures of alveoli and respiratory bronchioles form the acinus (Dunnill, 1962), the unit of gas exchange of the lung (Figure 1.2). The type of emphysema seen is determined by the area of the lung, or the area within the acinus, which is damaged.

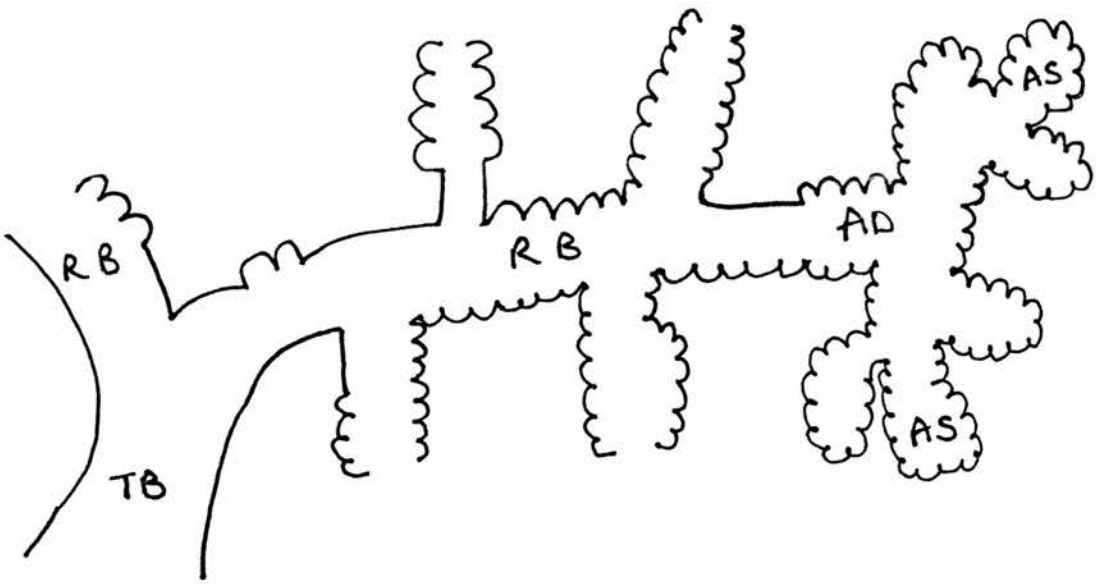


Figure 1.2 Normal architecture of the lung showing the series of branching airways ending in the respiratory airways (respiratory bronchioles (RB), alveolar ducts (AD) and alveolar sacs (AS)) which comprise the acinus. TB refers to the terminal bronchiole.

1.1.3 Morphological variants of emphysema

1.1.3.1 Centriacinar emphysema

Centriacinar emphysema is the most common form of emphysema, and is more closely associated with cigarette smoking than panacinar emphysema (Gillooly & Lamb, 1993b). The lesions of centriacinar emphysema are found centred around the respiratory bronchioles, while the remainder of the acinus, the alveolar sacs and alveolar ducts, are normal, Figure 1.3. This form of emphysema appears on the cut surface of the lung as focal lesions of damage surrounded by normal lung parenchyma (Lamb, 1995). It tends to be more common, and more severe, in the upper lobes of the lungs, and towards the apex of the lobe (Thurlbeck, 1963). In very severe centriacinar emphysema, the destruction of lung tissue may become confluent, involving the periphery of the acinus, making it difficult to distinguish from panacinar emphysema (Lamb, 1995).

1.1.3.2 Panacinar emphysema

Panacinar emphysema is diagnosed where enlarged airspaces are evenly distributed across the acinus (Lamb, 1995), Figure 1.3. Generally, panacinar emphysema does not affect the lung in focal lesions as does centriacinar emphysema, rather panacinar damage occurs throughout the affected region, with the same degree of severity. In panacinar emphysema, the destruction of airspace walls results in simplification of the architecture of the lung, with the distinction between respiratory bronchioles and alveolar sacs and ducts being lost (Dunnill, 1987). The lower lobes of the lung are preferentially involved

in panacinar emphysema (Thurlbeck, 1963), and severity tends to increase towards the base of the lobes. Panacinar emphysema is the form of emphysema associated with alpha-1 antiprotease deficiency (Guenter *et al*, 1968), indicating the likely importance of systemic rather than just local factors in the pathogenesis of this disease.

1.1.3.3 Paracicatricial emphysema

As its name implies, paracicatricial emphysema is invariably seen in association with scarring. It does not involve any particular part of the acinus or the lung, but is focal to the region of scarring (Dunnill, 1987). Generally, this type of emphysema is asymptomatic since it does not involve large areas of lung tissue (Lamb, 1995). The pathogenesis of this form of emphysema is thought to be increased elastic pull on surrounding alveoli from scarring, and the inflammation and fibrosis around the scar narrow the bronchioles such that air is trapped in the alveoli, further distending and breaking the alveolar walls (Dunnill, 1987).

1.1.3.4 Paraseptal emphysema

Paraseptal emphysema is the form of emphysema in which the alveolar sacs and ducts of the acinus are preferentially involved, while the respiratory bronchioles remain uninvolved (Lamb, 1995), Figure 1.3. It is most apparent at the periphery of the lobules, proximal to the lobular septa and adjacent to the pleura, and is generally more severe in the upper lobes of the lung. In common with paracicatricial emphysema, paraseptal emphysema is often found adjacent to areas of scarring and fibrosis.

The different morphological forms of emphysema often coexist in the same lung sample (Saetta *et al*, 1994b). This can make pathological diagnosis difficult, particularly where severe damage is present.

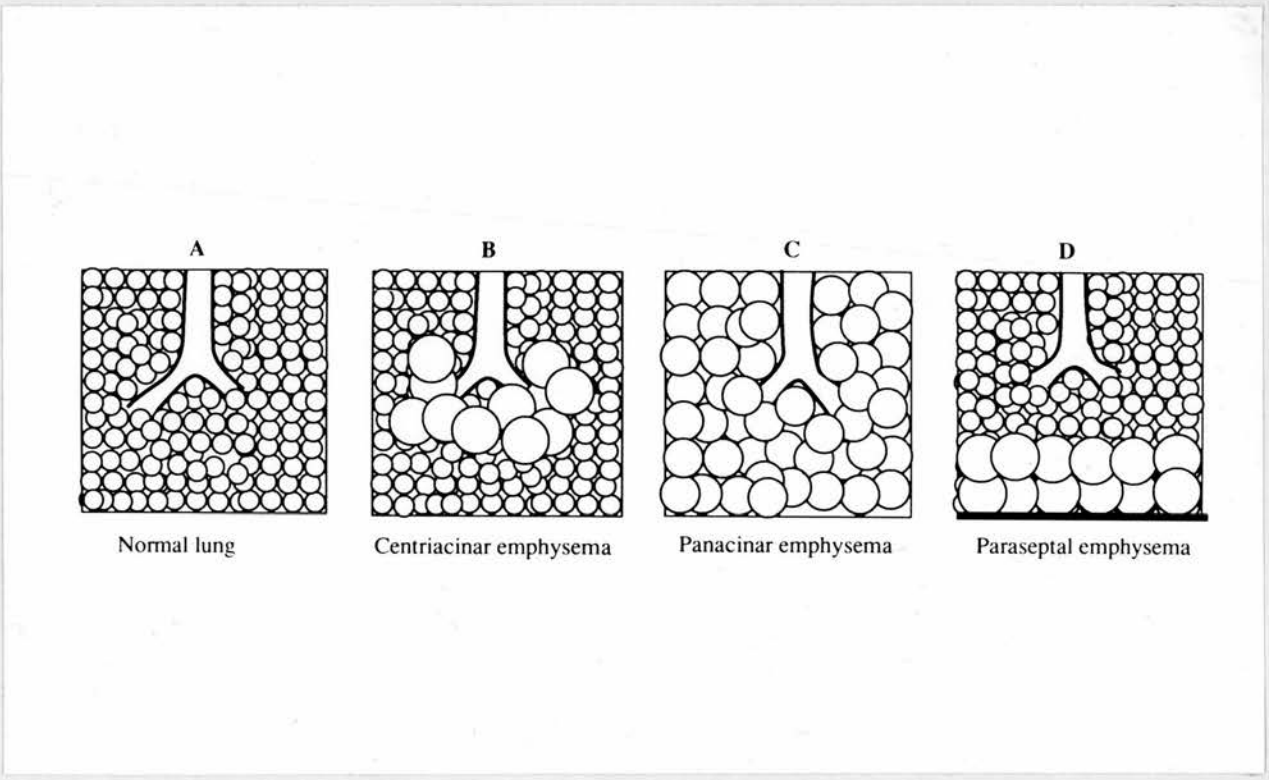


Figure 1.3 Diagrammatic representation of emphysema patterns in human lung.

1.1.4 Morphological assessment of emphysema

There are a number of methods by which emphysema can be measured in lung samples. These can be divided into either macroscopic assessment or microscopic assessment techniques, and both techniques have advantages and disadvantages.

1.1.4.1 Macroscopic assessment of emphysema

Macroscopic assessment involves the examination by eye of inflated lung tissue, with abnormal airspaces being classified as those airspaces measuring 1mm diameter or more. Several methods have been reported in the literature for the macroscopic assessment of emphysema (Lamb, 1995). The macroscopic assessment techniques described in the literature allow either severity or involvement to be assessed in a manner which is more accurate than evaluation of the entire lung by eye. However, true quantitative measurement is not possible using any of these techniques.

Automated techniques for macroscopic assessment have been developed which allow unbiased quantitation. The automative methods described use light transmission through lung sections to detect differences between normal lung and less dense emphysematous lung (Kory, 1966; Longfield, 1966).

Although truly quantitative, these methods are still subject to disadvantages. Differences in the thickness and pigmentation of lung sections measured will affect both the density and hence the light transmission of the sample. Only large amounts

of lung destruction may be detected using light transmission and mild disease may remain undetected.

There are several disadvantages of macroscopic assessment techniques. Firstly, they do not allow pattern of disease to be assessed. This must be done separately, and when more than one form of emphysema is present, separate evaluation of severity and/or involvement must be carried out. Secondly, macroscopic assessment only detects airspaces of 1mm diameter or more. The size of a normal airspace is approximately 250 μm (Schreider & Raabe, 1981), hence macroscopic detection occurs at a late stage in the disease process where 75% of normal tissue has been destroyed. The use of macroscopic assessment precludes the study of early emphysema where little damage has occured. Macroscopic detection will also not identify increased airspace size in the cohort of cases with airspaces measuring between 250μm and 1mm, hence the number of emphysema cases will be limited, and biased towards severe disease.

1.1.4.2 Microscopic measurement of emphysema.

In order to detect early damage occuring in lungs before the stage where it can be visualized macroscopically, several techniques have been developed which can detect emphysema using microscopes (Saetta *et al*, 1985; Saito *et al*, 1989; Nagai *et al*, 1989). However, microscopic techniques are open to subjective interpretation and they may be non-quantitative (Gillooly *et al*, 1991). The mean linear intercept (Lm) is often used as a microscopic measure of the alveolar surface area. While the Lm is an objective

measurement of the loss of alveolar tissue in microscopic emphysema, there is little information available on baseline levels of Lm measurements in the general population. Furthermore, unless automated techniques employing image analysis are used, the production of Lm measurements is time-consuming and labour intensive (Gillooly *et al*, 1991). Development of the Fast Interval Processor, an automated image analysis system which measures the airspace wall surface area per unit volume of lung tissue (AWUV) by Gillooly and colleagues (1991) has enabled the limits of normality of AWUV to be measured in the general population to establish a baseline for airspace size in relation to age (Gillooly & Lamb, 1993a). This work has provided a basis for the microscopic measurement of emphysema in smokers, and it has subsequently been shown that cigarette smoking is not the major influencing factor governing susceptibility to microscopic emphysema (Gillooly & Lamb, 1993c).

1.1.5 Aetiology and pathogenesis of emphysema

Emphysema is associated with cigarette smoking, but the reason why cigarette smoke exposure results in destruction of lung tissue is unclear. There is little direct evidence to causally link cigarette smoke exposure with emphysema. In attempting to elucidate the manner by which cigarette smoke results in emphysema, 2 main theories, the

protease/antiprotease imbalance theory and the oxidant/antioxidant theory, have been proposed.

1.1.5.1 Protease/Antiprotease theory of emphysema

Much of the evidence to support the protease/antiprotease theory is based on the finding that emphysema can be induced in the lungs of animals by installation of proteases, coupled with evidence that deficiency in α1-antiprotease results in increased susceptibility to emphysema. This evidence led Janoff & Carp to propose the protease/antiprotease theory in 1977 (Janoff & Carp, 1977a), and to this date, this hypothesis remains the major focus of investigation into the aetiology of emphysema.

1.1.5.2 α1 Antiprotease deficiency

Deficiency of α1-antiprotease (α1 anti-trypsin, α1Pi) was first recognized to be associated with susceptibility to emphysema by Laurell and Eriksson in 1963. Deficiency of α1Pi results as a consequence of polymorphism of the gene encoding the enzyme. α 1Pi is the major antiprotease circulating the body in the serum. Production of α1Pi occurs primarily in the liver. The function of α1Pi is the inhibition of the proteases elastase, collagenase, trypsin and thrombin (Kueppers & Bearn, 1966), however, elastase is thought to be the major physiologic substrate of α1Pi since it inactivates elastase faster than any other protease (Weissler, 1987). α1Pi inactivates proteases by acting as a false

substrate. Proteases recognize the active site of α1Pi, a Methionine-Serine peptide bond at amino acid postion 358, and covalently bind the antiprotease. It has been shown that the integrity of the Methionine site is essential for function of α1Pi (Johnson & Travis, 1979).

Deficiency of α1Pi can result from a variety of genetic defects, including gene deletion (Takahashi & Crystal, 1990), point mutations resulting in premature termination of transcription, and production of unstable mRNA (Satoh *et al*, 1988; Garver *et al*, 1986).

The major alleles of the α1Pi gene are the M, S, Z and null alleles. Individuals with normal α1Pi levels are homozygous for the M allele, and the frequency of this genotype in populations of Northern European extraction is 95% (Gadek & Crystal, 1982). Homozyotes for the ZZ allele show 12% of the normal α1Pi levels (Gadek & Crystal, 1982) and are present at an estimated frequency of 1 per 2500 in the US population (Weissler, 1987). Heterozygotes of the M, Z and S alleles, and homozygotes for the S allele show reduced α1Pi levels. MZ heterozygotes show a frequency of 4% in the US population (Weissler, 1987).

Deficiency of α1Pi predisposes individuals to develop emphysema, and this is specific to emphysema of panacinar pattern (Guenter *et al*, 1968). The exact frequency of the ZZ genotype in the general population is unknown since these individuals will only come to light if they experience symptoms of respiratory disease (Weissler, 1987). As previously mentioned, emphysema only becomes symptomatic when one third of the lung tissue has been destroyed (Pratt, 1988).

Conceivably there is a proportion of non-smoking ZZ individuals with no symptoms of emphysema which has never been included in studies of ZZ deficiency.

Smoking is very important in the induction of disease in α1Pi deficient individuals. The average age of onset of symptomatic respiratory disease in ZZ homozygotes who smoke is 40 years, as compared to 54 years in non-smoking ZZ individuals (Gadek & Crystal, 1982). Homozygotes for the ZZ allele of α1Pi show an average rate of decline in FEV1 which is approximately twice that of individuals with normal levels of α1Pi (Weissler, 1987).

While deficiency of α1Pi may increase the susceptibility of an individual to developing emphysema, ZZ individuals may only develop emphysema after smoking (Gadek & Crystal, 1982). Therefore simply an imbalance of proteases and antiproteases is insufficient to cause emphysema, and other crucial mechanisms must be involved in the aetiology of this disease.

1.1.5.3 Animal models of emphysema

Animal models have contributed significantly to the understanding of how an imbalance in proteases and antiproteases might produce tissue destruction which is similar to emphysema. The first animal model for emphysema was described in 1964 when Gross and colleagues introduced papain into the lungs of rats. The tissue destruction was shown to result from the elastolytic properties of the papain and subsequently, several studies demonstrated that emphysema could be induced in

animal lungs with the installation of pancreatic elastase. Whilst pancreatic elastase is a very potent protease, it is unlikely to be important in the development of pulmonary emphysema since it requires supra-physiological doses to produce emphysema by intravenous injection of elastases (Ip *et al*, 1980).

Inflammatory cells are a source of elastase and other proteases which may be relevant to the aetiology of emphysema. Proteases are used by these cells to facilitate their migration through connective tissues to reach sites of inflammation. Neutrophil elastase is a serine protease capable of degrading fibronectin, elastin, collagen types III and IV, and proteoglycans. The elastase is produced by promyelocytes and stored in the azurophilic granules of mature neutrophils. Studies by Fonzi & Lungarella (1979) and Tarjan and colleagues (1980) have shown that intratracheal instillation of homogenates of leukocytes causes emphysema in animal lungs. Subsequently, studies using purified human neutrophil elastase have produced emphysema when instilled intratracheally into the lungs of animals (Janoff *et al*, 1977b; Senior *et al*, 1977; Snider *et al*, 1984), and purified dog neutrophil elastase has produced emphysema upon intratracheal instillation to dogs (Sloan *et al*, 1981).

Upon intratracheal installation of elastase to hamster lungs, within one hour oedema, haemorrhage and infiltration of inflammatory cells is seen (Lucey & Clark, 1982). Air space enlargement occurs over the next few hours, and 24 hours following elastase introduction both elastin and collagen levels in the lung will have been reduced. Elastin may be depleted by as much as two thirds of its original content (Karlinsky & Snider, 1978).

Elastase has been shown to be cleared from the lungs to the pulmonary circulation 24 hours after instillation (Stone *et al*, 1977), however damage continues to occur, with the production of stable emphysematous lesions occurring at approximately 21 days post insult (Weissler, 1987). Studies of the breakdown products of elastin indicate that destruction is maintained for 2 weeks following elastase instillation (Kuchich *et al*, 1980; Janoff *et al*, 1983). It is possible that some of this destruction is mediated by neutrophil elastase which has been ingested by macrophages and released more slowly as the macrophages die or undergo phagocytosis (Weissler, 1987).

1.1.5.4 Relevance of emphysema induced by intratracheal instillation of proteases in animals

Although intratracheal instillation of proteases to animal lungs can produce emphysema, attempting to apply this information to produce a theory of the aetiology of human emphysema is problematic. Firstly, a single dose of elastin is enough to produce emphysema in an animal lung, whereas in humans, it takes on average 30 years to develop symptomatic emphysema (Weissler, 1987). Secondly, intratracheal instillation of proteases is not an ideal model of a disease thought to evolve from cigarette smoke induced damage and recruitment of leukocytes, and the subsequent local release of proteases.

Several studies have attempted to produce more physiological models of human emphysema by firstly exposing animals to cigarette smoke, secondly, by the introduction of compounds to increase the sequestration of leukocytes in the lungs, and finally, by the utilisation of agents which damage or reduce the activity of α1-antiprotease.

1.1.5.5 Animal models of emphysema produced by cigarette smoke exposure

Exposing animal lungs to cigarette smoke in order to produce emphysema is inherent with difficulty, and while many smoking studies have been carried out, they have for the most part failed to produce emphysema (Snider *et al*, 1986). Species differences provide a major problem. Because human emphysema takes, on average, decades of smoking to develop, precise dosage reproduction is difficult. Moreover, many experimental variables exist, such as differences in experimental technique in delivering cigarette smoke, in strength of cigarettes used, and in length of exposure times, perhaps explaining why much of the research carried out using animals has yielded conflicting results. Where morphologic changes are generated, often insufficient study of the qualitative and quantitative evidence of emphysema is undertaken to conclude whether the lungs manifest all the changes seen in human emphysema (Huber *et al*, 1981).

Often, studies which have exposed animal lungs to chronic cigarette smoke in order to produce models of emphysema have yielded histopathology which resembles other

human lung diseases. For example, beagle dogs have been exposed to 2-7 cigarettes daily over 2-4 months, which resulted in airspace enlargement. This airspace enlargement was not comparable to human emphysema however, since the enlargement appeared to result from the deletion of interalveolar septa, and expansion of interalveolar pores, rather than from destruction of the parenchyma (Frasca *et al*, 1983). In rats and hamsters, cigarette smoke exposure has been shown not to cause emphysema, but rather to generate pathology similar to that seen in human respiratory bronchiolitis, that is, epithelial hyperplasia, macrophage accumulation, and alveolar wall fibrosis (Bernfield *et al*, 1979; Dalbey *et al*, 1980; Wehner *et al*, 1981). These findings suggest that cigarette smoke exposure induces different functional changes in the lungs of dogs, rats and hamsters as compared to the damage seen in human emphysema, and these differences may represent a different underlying pathogenetic mechanism.

In perhaps the most comprehensive study of an animal model of emphysema induced by chronic cigarette smoke exposure, Huber and colleagues (1981) exposed approximately 300 rats thrice daily to cigarette smoke over a 6 month period. Reduction of parenchyma in the region distal to the terminal bronchioles of the lung was demonstrated. Changes in the physiological function of the lungs indicating a reduction in lung elastic recoil were also observed, adding support to the possibility that this study provides an animal model of emphysema. However, as the authors point out, their study did not indicate whether or not the increased airspace size demonstrable was permanent, nor could they ascertain whether the airspace

enlargement was the result of destruction, or was a reflection of differences in lung growth of the smoke exposed rats.

In conclusion, research to produce a convincing animal model to study the pathogenesis of human emphysema using chronic cigarette smoke exposure has failed.

1.1.5.6 Animal models investigating the effect of protease/antiprotease imbalance

Several studies investigating imbalances in proteases and antiproteases in animal lungs have been carried out. By exposing rats to puffs of cigarette smoke, Janoff and colleagues (1979) were able to show a reduced ability of lavage fluid α1Pi to inhibit elastase. Activity of α1Pi in lavage fluid has also been reduced significantly by the addition of human neutrophil myeloperoxidase and either hydrogen peroxide or glucose oxidase and glucose to hamster lungs (Zaslow *et al*, 1985).

To produce sequestration of leukocytes in the lungs of animals, several investigators have injected endotoxin intravenously in rhesus monkeys (Wittels *et al*, 1974), and dogs (Guenter *et al*, 1981), and this has been shown to result in mild emphysema. D-galactosamine is a compound which reduces α1-antiprotease levels in the serum of rats by damaging the liver, and hence reducing α1Pi synthesis by hepatocytes (Blackwood *et al*, 1979). Combination of D-galactosamine with endotoxin injection

has been shown to decrease lung elastin content, and causes some physiological effects on lung function (Blackwood *et al*, 1984).

Chloramine T is an oxidizing agent which has been shown to cause reduction in the activity of serum α1Pi in dogs, and which has been used to generate lesions similar to emphysema in the lungs of dogs (Abrams *et al*, 1981).

1.1.5.7 Genetic models of emphysema

Genetic models of emphysema have been reported in the literature. The blotchy mouse is a model of deficient copper transport, which progressively develops lesions similar to panacinar emphysema. Production of mature elastin and collagen requires cross-linking of precursors by a copper-dependant enzyme lysyl oxidase (Weissler, 1987). The deficiency in copper transport demonstrable in the blotchy mouse affects lysyl oxidase activity such that elastin and collagen synthesis is decreased (Snider *et al*, 1986). The tight-skinned mouse is another genetic model of emphysema (Szapiel *et al*, 1981; Rossi *et al*, 1984) which demonstrates deficiency of α1Pi in the serum, although liver mRNA levels are normal (Martorana *et al*, 1993). The tight-skin mouse develops alveolar septal destruction with lesions becoming apparent at 8 months of age, and air space enlargement developing from the age of 12 months (Martorana *et al*, 1993). Tight-skin mice have also been reported to have abnormally high elastase and cathepsin G levels in the lysosomal secretions of their neutrophils (Gardi *et al*, 1994). The tight-skin mouse lung has been shown to contain increased levels of inflammatory cells in the absence of infection (Snider *et al*, 1986).

1.1.6 Mechanism of lung tissue destruction in emphysema

Studies carried out on animals have provided important information concerning how tissue destruction may occur. However, even the more physiological studies which mimic the long onset, genetic susceptibility, and possible involvement of polymorphonucleocytes, do not provide any direct evidence for a link between cigarette smoke exposure and emphysema. How then can the information gained from animal model research be used to speculate on the manner in which cigarette smoking causes the tissue destruction resulting in emphysema?

1.1.6.1 Neutrophil elastase

Neutrophil elastase is a serine protease which is stored in the azurophilic granules of the neutrophil. It attacks almost all of the structural proteins of the lung, including elastin, fibronectin, proteoglycans, and collagen types III (Mainardi *et al*, 1980) and IV (Agar *et al*, 1986; Aruoma & Halliwell, 1989). The release of neutrophil elastase in healthy lung tissue is normally kept to a minimum because few neutrophils are found in lung tissue unless inflammatory cell recruitment occurs to increase their numbers. Hunninghake and colleagues (1979) reported that neutrophils, eosinophils and basophils make up less than 1% of the cells found in bronchoalveolar lavage, and this reflects the numbers of polymorphonuclear leukocytes in healthy alveoli.

Smokers lungs however, have much higher proportions of inflammatory cells in both bronchoalveolar lavage fluid (Reynolds & Newball, 1974; Warr & Martin, 1976) and in their respiratory bronchioles and alveoli (Niewoèhner *et al*, 1974; Kuhn & Senior, 1978). These cells are thought to be recruited to the lung by chemotaxis in a number of ways. Nicotine has been shown to be chemotactic for neutrophils, and to increase the responsiveness of neutrophils to the chemotactic C5a complement peptide (Totti *et al*, 1984). The inflammatory response to tissue injury from cigarette smoke components is also thought to result in chemotaxis. Stimulated alveolar macrophages have been shown to release chemotactic factors for neutrophils *in vitro* following activation by immunoreactive particles (Merrill *et al*, 1980; Hunninghake *et al*, 1980). An aqueous suspension of whole cigarette smoke has been shown to modify the C3 component of complement such that the alternative complement pathway is activated *in vitro* (Kew *et al*, 1985), which may contribute to the recruitment of inflammatory cells to the lung.

1.1.6.2 Involvement of other proteases in emphysema

Neutrophils are not the only cells implicated in the release of proteases which may cause lung tissue damage. Monocytes contain an elastase which, although only 3% as much as present in neutrophils, may be expressed on the cell surface (Weissler, 1987), and may therefore be more accessible to mediate damage (Senior, 1982). Mouse macrophages secrete a metalloproteinase (Werb & Gordon, 1975) which can degrade elastin, and which has been shown to be capable of proteolysis of α1Pi (Banda *et al*, 1980). Furthermore,

metalloelastases are not susceptible to inactivation by α1Pi (Weissler, 1987). In humans, bronchoalveolar lavage (BAL) fluid of smokers contains a metalloprotease, the amount of which is correlated negatively with decreased activity of α1Pi (Niederman *et al*, 1984). Macrophages contain other proteases which may have roles in protease/antiprotease imbalances. Cathepsin B has elastolytic properties, and is expressed on the macrophage cell surface (Orlowski *et al*, 1981). The macrophage cell surface also expresses plasminogen activator, which can activate plasminogen to plasmin, a very effective degrader of fibrin and proteoglycans (Chapman *et al*, 1984). Mass and colleagues introduced homogenates of alveolar macrophages to the lungs of dogs by aerosol, and observed that this treatment produced mild emphysema (1972). As well as containing proteases which can be released and contribute to tissue damage, macrophages can be primed to enhance their release of oxygen radicals by proteases (Speer *et al*, 1984).

Neutrophils contain proteases other than elastase which can be destructive to lung tissues. Neutrophil collagenase causes little damage (Karlinsky & Snider, 1978), presumably because it is specific only for Type I collagen. Cathepsin G is capable of degrading elastin, but at 12% of the rate of digestion mediated by elastase. Proteoglycans, fibronectin and laminin are also potential substrates of Cathepsin G (Kao *et al*, 1988). However, Lucey and colleagues (1985) were unable to produce emphysema by intratracheal instillation of Cathepsin G to the lungs of hamsters. Some evidence has been presented which suggests that Cathepsin G may have a role in the solubilization of lung elastin by neutrophil elastase in humans (Boudier *et al*, 1981; Reilly *et al*, 1984; Lucey *et al*, 1985). Proteinase 3 has

elastolytic properties, and is the only protease besides neutrophil elastase to induce emphysema-like changes in animals (Kao *et al*, 1988). However, this finding has not been confirmed. Neutrophil myeloperoxidase has been shown to produce lesions similar to pulmonary fibrosis when instilled into the lungs of animals (Johnson & Ward, 1981).

The research which has been carried out on the possible involvement of inflammatory cells in the aetiology of emphysema is interesting but inconclusive. Inflammatory cells do contain proteases which are capable of lung tissue degradation, and there is some evidence to support the recruitment of inflammatory cells to the lung following cigarette smoke exposure. However, there is, as yet, no conclusive proof that emphysema results from an imbalance in the levels of proteases and antiproteases which is triggered by cigarette smoking.

1.1.6.3 Cigarette smoke involvement in emphysema

Emphysema may develop following cigarette smoke exposure as a direct result of toxicity from components in the smoke, either directly causing cytotoxicity, or indirectly by affecting the protease/antiprotease levels in the lungs. Research into the mechanisms by which reactive species in cigarette smoke might cause tissue destruction can be referred to as the oxidant/antioxidant theory of emphysema aetiology.

Cigarette smoke contains 10^{16} oxidants per puff, (Pryor *et al*, 1986). These reactive species are capable of damaging tissue directly, and may contribute to the protease burden of lungs by inactivating α1Pi in a number of ways.

The gas phase of cigarette smoke contains alkoxyl radicals, peroxyl radicals and alkyl radicals (Evans & Pryor, 1994), Figure 1.4. Reaction between these radicals and nitric oxide and nitrogen dioxide also contained in cigarette smoke (Church & Pryor, 1985) can produce further harmful species, including peroxynitrates (Evans & Pryor, 1994) and peroxynitrites (Beckman *et al*, 1994; Koppenol *et al*, 1992). Nitric oxide can react with peroxyl radicals and produce alkyl peroxynitrites (Padmaja & Huie, 1993), which are cytotoxic. It is thought that a steady state production and destruction of radicals occurs in cigarette smoke, since the half-life of the radicals present in smoke is long (Church & Stone, 1985). The gaseous phase of cigarette smoke is capable of causing nitration of tyrosine, probably through reactions of nitrogen oxides (Eiserich *et al*, 1994).

The particulate phase, or tar, of cigarette smoke contains 4 radicals, and phenolics, which can autoxidize and polymerize to form semiquinone radicals (Evans & Pryor, 1994). The major tar radical is a quinone/hydroquinone complex which may reduce molecular oxygen to produce the superoxide radical, capable of generating hydrogen peroxide and the hydroxyl radical (Church & Pryor, 1985), Figure 1.4. Aqueous

extracts of tar produce solutions that consume oxygen and contain hydrogen peroxide, superoxide radicals and hydroxyl radicals (Evans & Pryor, 1994).

The major organic-free radicals in gas-phase cigarette smoke are the alkyl radicals ($^{\cdot}R$) and the alkoxyl ($RO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals. A steady state production of radicals in cigarette smoke was suggeted by Church and Pryor (1985). In this model nitric oxide is slowly oxidised to nitrogen dioxide:

$$^{\cdot}NO_2 + 1/2\ O_2 \rightarrow {}^{\cdot}NO_2$$

Nitrogen dioxide may then react with reactive species, for example olefins, aldehydes and dienes, to produce a carbon centered radical, $R^{\cdot}$:

$$^{\cdot}NO_2 + C{=}{=}C \rightarrow O_2N{-}\underset{|}{\overset{|}{C}}{-}\underset{|}{\overset{|}{C}}{}^{\cdot}$$

$(R^{\cdot})$

In the presence of dioxygen, the $R^{\cdot}$ radical can rapidly form peroxyl radicals:

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$$

The peroxyl radical can react with nitric oxide to produce peroxynitrites:

$$ROO^{\cdot} + {}^{\cdot}NO \rightarrow ROO{-}NO$$

Alternatively, the peroxyl radical can react with $^{\cdot}NO_2$ to produce peroxynitrates:

$$ROO^{\cdot} + {}^{\cdot}NO_2 \rightarrow ROO{-}NO_2$$

Figure 1.4 Figure showing some of the major free radicals present in cigarette smoke and their reaction products which may result from a steady state production of radicals (Evans & Pryor, 1994).

1.1.6.4 Tissue damage from reactive oxygen species

Reactive oxygen species are directly damaging to tissues in a number of ways. Cigarette smoke can oxidise thiols (Fenner & Braven, 1968), and Church & Pryor (1985) suggest that nitric oxide and nitrogen dioxide in cigarette smoke can oxidise sulfhydryl groups, and thus impair enzyme function. Glutathione, an important antioxidant, has also been shown to be oxidised by nitric oxide and nitrogen dioxide (Church & Pryor, 1985). Alkyl peroxynitrates and peroxyl radicals are cytotoxic species (Padmaja & Huie, 1993; Pryor & Stone, 1993), as are nitrogen dioxide, peroxynitrites and peroxynitrates (Eiserich *et al*, 1994). Peroxynitrites are very powerful oxidizing agents to -SH groups (Radi *et al*, 1991). Nitrogen oxides also can cause the nitration of tyrosine (Eiserich *et al*, 1994) and of lysine ε-amino groups (Hood, 1993) and can produce peptide bond cleavage. In the lung, protein damage may impair the function of membrane transport and receptor proteins, antioxidants and surfactant proteins, and cause inactivation of important enyzmes, including α1Pi. Nitration of tyrosine might also affect tyrosine phosphorylation pathways of cell signalling (Eiserich *et al*, 1994).

Free radicals can oxidize lipids and proteins in biomembranes (Niki *et al*, 1993), and cigarette smoke can cause lipid peroxidation in plasma (Frei *et al*, 1991). Large amounts of lipid peroxidation can alter the fluidity, potential and permeability of membranes, which may lead to the eventual rupture of the cell membrane (Halliwell & Gutteridge, 1990). Moreover, some of the end-products of lipid peroxidation can be cytotoxic, affecting macrophage action, protein synthesis, and enzyme function, and mediating protein cross-linking, thrombin release, and chemotaxis of inflammatory cells (Halliwell & Gutteridge, 1990).

Lannan, and colleagues (1994) have shown that whole and gas phase cigarette smoke can cause increased cell detachment and lysis and decreased cell attachment and proliferation. When type I epithelial cells are damaged, type II cells replace them by proliferation and differentiation. Decreased proliferation and attachment of type II alveolar cells may contribute to the alveolar destruction seen in emphysema.

Superoxide produced by cigarette smoke has been shown to cause decreased membrane fluidity of inflammatory cells (Tsuchiya *et al*, 1993). Biologically, this may reduce the deformability of the inflammatory cells as they enter the lung, either in the pulmonary circulation or in the alveoli (Dye & Adler, 1994). Exposure of neutrophils to whole and gas-phase cigarette smoke has been shown to reduce deformability via disruption of F-actin turnover (Drost *et al*, 1992). This will slow

the passage of these cells out of the lung, hence any destructive effects mediated by inflammatory cells may be prolonged. In cells of the lung, decreased fluidity of the membranes will reduce cell spreading, which is necessary for cell attachment. Since cell attachment is required for proliferation, this will affect the ability of the lung to repair damage (Lannan *et al*, 1994).

Lung epithelial and endothelial permeability is increased in smokers as compared to the lung permeability of non-smokers (Dye & Adler, 1994). This is thought to be due to effects of cigarette smoke on the cell cytoskeleton. Cigarette smoke also has deleterious effects on the number of gap junctions (van der Zandt *et al*, 1990) and intercellular communication between gap junctions (Rutten *et al*, 1988).

Smokers have increased levels of ferritin in their lavage fluid (McGowan & Henley, 1988), and in alveolar macrophages (Quan & Golde, 1981). The binding of iron to ferritin can prevent free iron from participating in hydroxyl radical production and lipid peroxidation (Heffner & Repine, 1989). However, cells which are damaged following inflammation can release their ferritin-bound iron (Halliwell & Gutteridge, 1984), and following interaction with superoxide, organic radicals, and ascorbate, the iron can be freed to contribute to toxicity (Gutteridge *et al*, 1980; Bannister *et al*, 1982; Biemond *et al*, 1984; O'Connell *et al*, 1986). Lung tissue from patients with suspected pathogenetic excess of reactive oxygen species demonstrates sequestration

of erythrocytes and platelets in the microvasculature of the lung. Erythrocytes and platelets can contribute to oxidative damage. Erythrocytes release iron bound to hemoglobin which can then participate in iron-catalysed free radical reactions (Cross *et al*, 1987). Platelets are capable of releasing chemotactic factors for neutrophils (Boogaerts *et al*, 1982), and can stimulate increased neutrophil aggregation, granule release (Boogaerts *et al*, 1982) and adherence (Tzeng *et al*, 1984). Furthermore, platelets which have been stimulated with thrombin can directly damage endothelial cell cultures (Jørgenson *et al*, 1986). Platelets can also release endoperoxides which neutrophils can ingest and metabolize to produce inflammatory products (Marcus *et al*, 1984).

As well as being directly damaging to lung tissues, oxidants derived from cigarette smoking can cause tissue destruction indirectly. Many components of cigarette smoke can inactivate α1Pi and it is generally believed that this occurs primarily by oxidation of the protein (Evans & Pryor, 1994). Besides the methionine residue in the active site (Met^{358}), α1Pi has several other methionine residues which can be oxidized thereby affecting protein function (Evans & Pryor, 1994). Hydrogen peroxide in either the gas phase or tar components of cigarette smoke can inactivate α1Pi. Hydroxyl radicals and superoxide may inactivate α1Pi by direct oxidation. Alkyl radicals do not inactivate α1Pi, which may be due to their high reactivity - they may be so reactive that they interact with other substrates before reaching α1Pi. Transition metals in cigarette smoke can participate in the production of hydrogen

peroxide. α1Pi is known to have at least 1 copper binding site, and ferric ions can bind α1Pi and produce reactive, peroxide derived species which can damage the protein. Cigarette smoke contains high levels of both iron and copper. In lower concentrations, nitrogen dioxide and hydrogen peroxide may cause slow damage to α 1Pi. Peroxynitrous acid, formed from reaction of nitric oxide with superoxide may also have damaging affects on α1Pi. Inactivation of α1Pi can occur by protein damage, through nitration of tyrosine residues and ε-amino groups of lysine, and through peptide bond cleavage, all of which can be produced by nitrogen oxides (Evans & Pryor, 1994; Eiserich *et al*, 1994).

Exposure of the lungs to oxidants in cigarette smoke not only affects the balance of proteases and antiproteases through the inactivation of α1Pi. Fligiel and colleagues (1984) showed that the exposure of proteins to hydrogen peroxide increased their susceptibility to proteolytic attack.

Cigarette smoke may also be involved in the pathogenesis of emphysema through the inhibition of the repair of damaged elastin. Elastin is formed by the cross-linking of tropoelastin by desmosine. Before this cross-linking can occur, lysine residues of elastin must be oxidised. This oxidation is mediated by the copper-dependant enzyme lysyl oxidase (Weissler, 1987). If this enzyme is inhibited, damage to the lung by elastase is greatly increased (Weissler, 1987). Cigarette smoke is known to

inhibit elastin synthesis by blocking the cross-linking of tropoelastin (Janoff, 1985). Basic cigarette smoke components can inhibit elastin synthesis which may occur by the addition of polyanions to elastin. Lysyl oxidase is a negatively charged molecule (Kagan *et al*, 1981), hence enzyme binding of elastin will be reduced if the elastin becomes more positively charged. Another effect of the addition of polyanions to elastin is increased attraction of elastase, which is positively charged (Kagan *et al*, 1981). Furthermore, lysyl oxidase function can be affected by reduced availability of copper, on which it is dependant. Copper malnutrition, for example, can increase the severity of damage induced by elastase (O'Dell *et al*, 1978). Cigarette smoke is known to contain copper chelators, which may remove copper required for lysyl oxidase mediated elastin cross-linking, and hence hinder repair (Weissler, 1987).

1.1.6.5 Environmental damage to lung tissue

Nitrogen oxides are environmental pollutants and are present in smog. Nitrogen dioxide can cause emphysema-like changes in animal lungs following long-term exposure (Snider *et al*, 1986). In hamsters nitrogen dioxide decreases lung elastin content and causes emphysema-like change, although in adults this requires very great levels of exposure (Snider *et al*, 1986).

1.1.6.6 Lung protection against oxidative attack

While oxidative attack on tissue exposed to cigarette smoke can produce damage which may result in emphysema, the lung is not unprotected against this form of destruction. The lung has a series of antioxidant mechanisms which serve to protect against oxidative attack. These mechanisms were defined by Sies (1987) as being any cell process which 1. blocks the formation of free radicals; 2. metabolizes oxidants to less harmful products; 3. compartmentalizes free radicals to prevent cell destruction; and 4. repairs damage caused by free radicals.

Major airways contain mucopolypeptide glycoproteins which can act as sacrificial scavengers of reactive species (Cross *et al*, 1984). The alveoli acquire many of their complement of antioxidants from the epithelial lining fluid. Alveoli have been shown to contain ceruloplasmin, transferrin, ascorbate, vitamin E, ferritin, bilirubin and methionine (Heffner & Repine, 1989). High levels of reduced GSH are also present in the alveoli (Cantin *et al*, 1987), and these levels are increased in smokers lungs. Bronchoalveolar lavage fluid recovered from normal individuals contains antioxidants which are capable of blocking lipid peroxidation *in vitro* (Pacht & Davis, 1988).

Superoxide dismutases catalyze the dismutation of superoxide to produce hydrogen peroxide and oxygen. The different superoxide dismutases are found primarily in the cytosol and nucleus, the mitochondria, or the plasma (Heffner & Repine, 1989).

Catalase catalyzes the dismutation of hydrogen peroxide to water and oxygen, and can remove both hydrogen peroxide and superoxide from the intracellular environment (Heffner & Repine, 1989). In mammalian cells, catalase is highly compartmentalized, primarily to the peroxisome (Chance *et al*, 1979).

The glutathione redox cycle is capable of reducing hydrogen peroxide, eliminating hydroperoxides (Ross *et al*, 1985) and lipid peroxides formed during free radical attack of membranes (Moldéus *et al*, 1986).

Compartmentalization to keep oxidants away from vulnerable cell structures is very important since oxidants are formed continuously in the cell during respiration. By compartmentalization to the mitochondria, any toxic species are kept from damaging the cell. Other examples of cell compartments which hold free radicals preventing damage are peroxisomes and microsomes (Heffner & Repine, 1989).

As has previously been mentioned, transitional metal ions are important in the production of oxidants, since they can enhance lipid peroxidation and participate in the production of the hydroxyl radical from superoxide and hydrogen peroxide. The hydroxyl radical is so very reactive and toxic to cells, in comparison to superoxide and hydrogen peroxide, that it is thought to be one of the most damaging species. By reducing the availability of free transitional metals, production of hydroxyl radicals can be limited (Heffner & Repine, 1987).

Free iron can be bound by the glycoproteins transferrin and lactoferrin, which transport iron in the circulation (Gutteridge, 1986a). Haemoglobin will readily release bound iron (Gutteridge, 1986b), but compartmentalization in the erythrocyte of haemoglobin prevents the iron from participating in free radical formation. Caeruloplasmin is a plasma glycoprotein which catalyses the formation of ferric iron from ferrous iron which can help produce free radicals (Heffner & Repine, 1989). Ferric iron can then be bound by transferrin. Caeruloplasmin can also scavenge superoxide and the hydroxyl radical, inhibit the oxidative inactivation of proteases by products of the leukocyte myeloperoxidase system, and bind copper, which can also participate in hydroxyl radical formation.

Vitamin E (α-tocopherol) is thought to be the primary defense against membrane damage by oxidants. Vitamin E converts superoxide, hydroxyl radicals and lipid

peroxyl radicals to less toxic forms, and can break the chain reaction of lipid peroxidation. It is lipid soluble and can integrate into lipid membranes where it can carry out antioxidant reactions (Heffner & Repine, 1989). β-carotene is a second lipid soluble antioxidant which is present in lipid membranes. β-carotene can scavenge superoxide, and can react with peroxyl-free radicals (Foote & Denny, 1968).

Vitamin C is a water-soluble antioxidant which is found in both intracellular and extracellular spaces. Scavenging of superoxide and hydrogen peroxide can be carried out by vitamin C (Heffner & Repine, 1989). Vitamin C can also inhibit the inactivation of antiproteases mediated by the neutrophil myeloperoxidase-halide system (Theron & Anderson, 1985), and inactivate oxidants released from neutrophils (Anderson *et al*, 1988).

Both vitamin C and β-carotene have pro-oxidative capabilities (Heffner & Repine, 1989). Vitamin C can convert ferric iron to ferrous iron, which can then participate in the generation of hydroxyl radicals (Halliwell & Gutteridge, 1984).

Uric acid is a potent antioxidant. It scavenges hydroxyl radicals, singlet oxygen, oxoheme oxidants, and peroxyl radicals (Heffner & Repine, 1989). Vitamin C oxidation can be prevented by uric acid (Sevanian *et al*, 1985), and transition metals

can be bound by uric acid (Davies *et al*, 1986), thereby removing them from availability to produce hydroxyl radicals.

ADP ribosyl transferase is a nuclear enzyme which mediates excision repair after minor or moderate damage to DNA (Schraufstätter *et al*, 1986), for example, single strand breaks. Following severe damage, the enzyme depletes cellular NAD+ and initiates cytotoxicity (Berger, 1985).

Phospholipase A2 and the seleno-enzyme phospholipid hydroperoxide glutathione peroxidase both degrade peroxidized membrane phospholipids therefore removing damaged membrane before chain peroxidation can be initiated (van Kuijk *et al*, 1987).

1.1.6.7 Other mechanisms of lung protection

Cellular proliferation to replace damaged tissue is another major mechanism of repair available to the lung. Rats exposed to hyperoxia show lung cell proliferation and changes in cell morphology which may be indicative of the production of cells which are more resistant to subsequent oxidant attack (Heffner & Repine, 1989).

As previously mentioned, Lannan and colleagues, 1994, found that decreased cell membrane fluidity, which can be induced by cigarette smoke exposure, affects the ability of cells to spread, attach and proliferate. Cigarette smoke exposure also caused increased cell detachment and lysis.

Lung protection can also be provided by recruitment of cells rich in antioxidants from the circulation. Erythrocytes, for example, contain high levels of superoxide dismutase (SOD), catalase, and GSH redox cycle components (Toth *et al*, 1984). Moreover, superoxide can cross the erythrocyte membrane and be dismutased by erythrocyte SOD (Lynch & Fridovich, 1978). Oxidant exposure causes erythrocytes to bind preferentially to endothelial cells (Wali *et al*, 1987), which may provide a sink for hydrogen peroxide and superoxide and prevent further reactive species formation (Winterbourn & Stern, 1987). Toth and colleagues, 1986, demonstrated that levels of GSH and catalase were higher in erythrocytes of smokers than in non-smokers. Smokers' erythrocytes were also more effective at protecting endothelial cell cultures against H_2O_2 damage when compared to the erythrocytes of non-smokers.

Platelets contain components of the glutathione redox cycle and catalase (Ramos Martinez *et al*, 1979; Del Principe *et al*, 1985). By virtue of their small size, platelets

can form close associations with activated neutrophils, allowing reduction of neutrophil-derived oxidants (Pietra *et al*, 1981).

Phagocytes also contain antioxidants, and can lower H_2O_2 levels *in vitro* (Berger *et al*, 1987). Phagocytes may be protective in the lung when unstimulated, or following cell lysis, since they may be sources of antioxidants (Heffner & Rapine, 1989).

1.1.7 Mechanisms of tissue destruction and pattern of disease

The mechanisms by which cigarette smoke may cause tissue damage have been discussed, but how might these mechanims contribute to destruction in such a manner that different patterns of damage result, for example producing centriacinar rather than panacinar emphysema in a lung sample?

The half-life of the hydroxyl radical has been estimated to be 10^{-9} seconds (Pryor, 1986), therefore hydroxyl radical mediated damage will occur close to the source of the hydroxyl radical (Leanderson, 1993). Following cigarette smoke exposure, the damage caused by the hydroxyl radical would be expected to occur around the region of the respiratory bronchioles. When cigarette smoke enters the lung, it enters progressively more numerous, smaller airways, which decreases flow of the smoke, until flow stops altogether in the region of the respiratory bronchioles. As the

particles contained in the smoke bounce around, they are deposited, and can be adsorbed onto the epithelium (Harley, 1988). Cigarette smoke components reach the alveolar ducts and sacs primarily by diffusion from the respiratory bronchioles (Saetta *et al*, 1994a). Consequently, the area of the lung most likely to be damaged as a direct result of cigarette smoking is the region of the respiratory bronchioles.

Centriacinar emphysema is the form of emphysema where damage is centred around the respiratory bronchioles. It is almost exclusively associated with cigarette smoking, and severity of disease is directly related to the dose of smoke exposure (Lamb, 1995). Panacinar emphysema, however, is found throughout the acinus, and is not as clearly related to cigarette smoke exposure as is centriacinar emphysema (Gillooly & Lamb, 1993b). Furthermore, panacinar emphysema is the form of disease seen in individuals with α1-antiprotease deficiency (Guenter *et al*, 1968). Alpha 1-antiprotease is a systemic enzyme, therefore it is possible that panacinar emphysema may be related to both the direct effects and the systemic effects of cigarette smoke exposure (Saetta *et al*, 1994a). In a recent article, Saetta and colleagues presented evidence that panacinar and centriacinar emphysema are different diseases, and that these 2 diseases give rise to the airflow limitation of chronic obstructive pulmonary disease by different mechanisms (Figure 1.5).

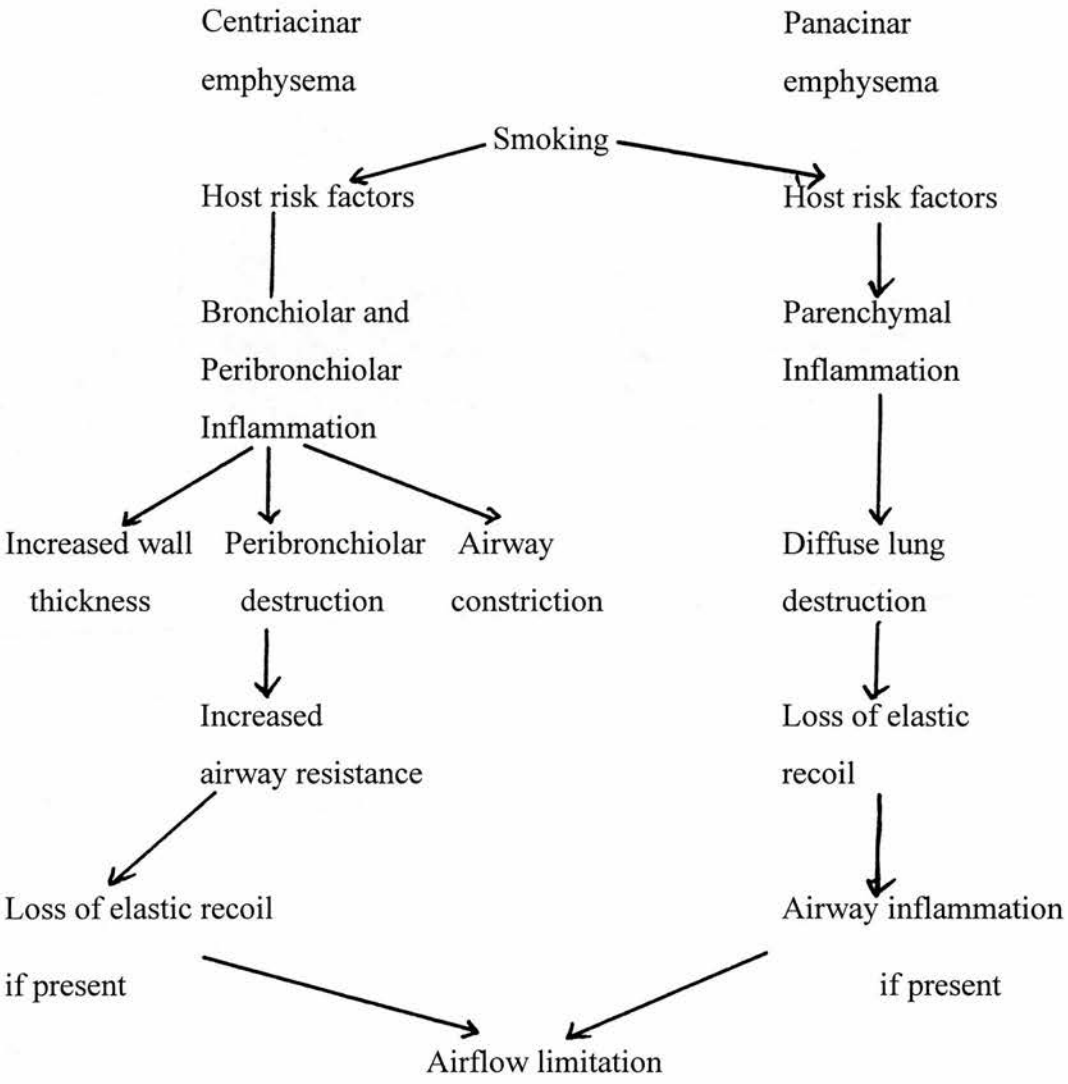


Figure 1.5 Diagram of the different mechanisms by which centriacinar and panacinar emphysema might give rise to chronic obstructive airways disease (adapted from Saetta *et al*, 1994a)

1.1.8 Emphysema and lung cancer

Emphysema and lung cancer are both cigarette smoking associated diseases. It is therefore probable that some components of cigarette smoke cause the damage which may result in emphysema or the genotoxic injury which eventually leads to lung cancer.

In the discussion of the possible role of oxidants in the pathogenesis of emphysema, it has been shown that oxidative species are cytotoxic. Oxidants have also been shown to be genotoxic. Cigarette smoke condensate can induce DNA single-strand breakage *in vitro* via the production of reactive oxygen species (Nakayama *et al*, 1985). The tar fraction of cigarette smoke has also been shown to induce DNA damage (Borish *et al*, 1985). Birnboim & Kanabus-Kaminska (1985) demonstrated that superoxide can cause DNA strand breakage, as can the hydroxyl radical (Bradely & Erickson, 1981). The hydroxyl radical can also cause the hydroxylation of DNA bases (Aruoma & Halliwell, 1989). Radical species may also demonstrate promoter activity (Church & Pryor, 1985).

Research into the aetiology of emphysema has generated more questions concerning the cause of emphysema instead of elucidating the basic mechanisms by which cigarette smoke can induce tissue destruction. Perhaps the most interesting question which arises from any discussion of the aetiology of emphysema is why do only

some smokers develop emphysema? Discounting genetic disorders such as α1-antiprotease deficiency and defects in copper metabolism, which would be expected to have a major impact on susceptibility to emphysema, some mechanism must exist which can vary interindividual susceptibility such that only a proportion of smokers develop emphysema. From discussion of the aetiology of emphysema, it is clear that this disease is polygenic, but even so, there must be some factor, or factors, with a great deal of influence on an individual's susceptibility. In cases of heavy smokers who do not develop emphysema one would predict a strong protective mechanism is present which can counteract the large quantities of toxic chemicals flooding the lungs. Equally, light smokers who are exposed to relatively small doses of cigarette smoke, and non-smokers, can develop emphysema, which suggests that these individuals have some fundamental defect in the protective capacity of their lungs which increases their risk of developing tissue destruction.

Gillooly & Lamb (1993b) measured the alveolar wall surface area per unit volume of lung tissue (AWUV) among smokers in order to study microscopic emphysema. When the smokers' mean AWUV values were plotted against the distribution of AWUV in a non-smoking population (Figure 1.5) (Gillooly & Lamb, 1993a), only 26% of smokers' AWUV values fell below the normal distribution, indicating the presence of microscopic emphysema, Figure 1.7. Furthermore, there was no correlation between daily cigarette consumption and low AWUV values. Of the heavy smokers, 69% had mean AWUV values within the normal distribution. The

finding that microscopic emphysema is present in only some smokers, and is not directly related to cigarette smoking in a dose-dependant manner, suggests that a proportion of individuals are inherently more susceptible to developing microscopic emphysema.

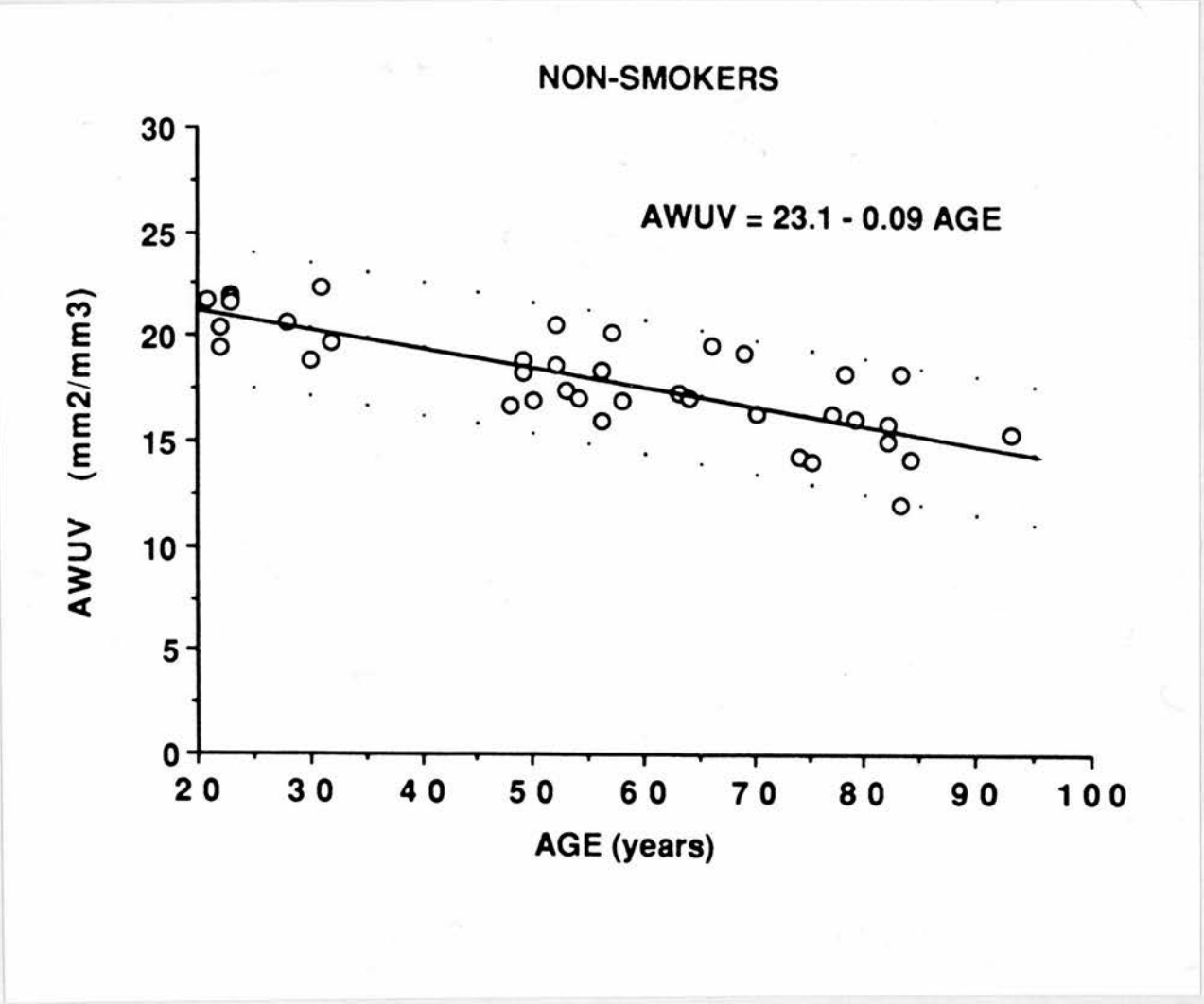


Figure 1.6 Graph showing the decline of alveolar wall surface area per unit volume of lung tissue (AWUV) with age. This data can be used to define the normal limits of AWUV in the general population.

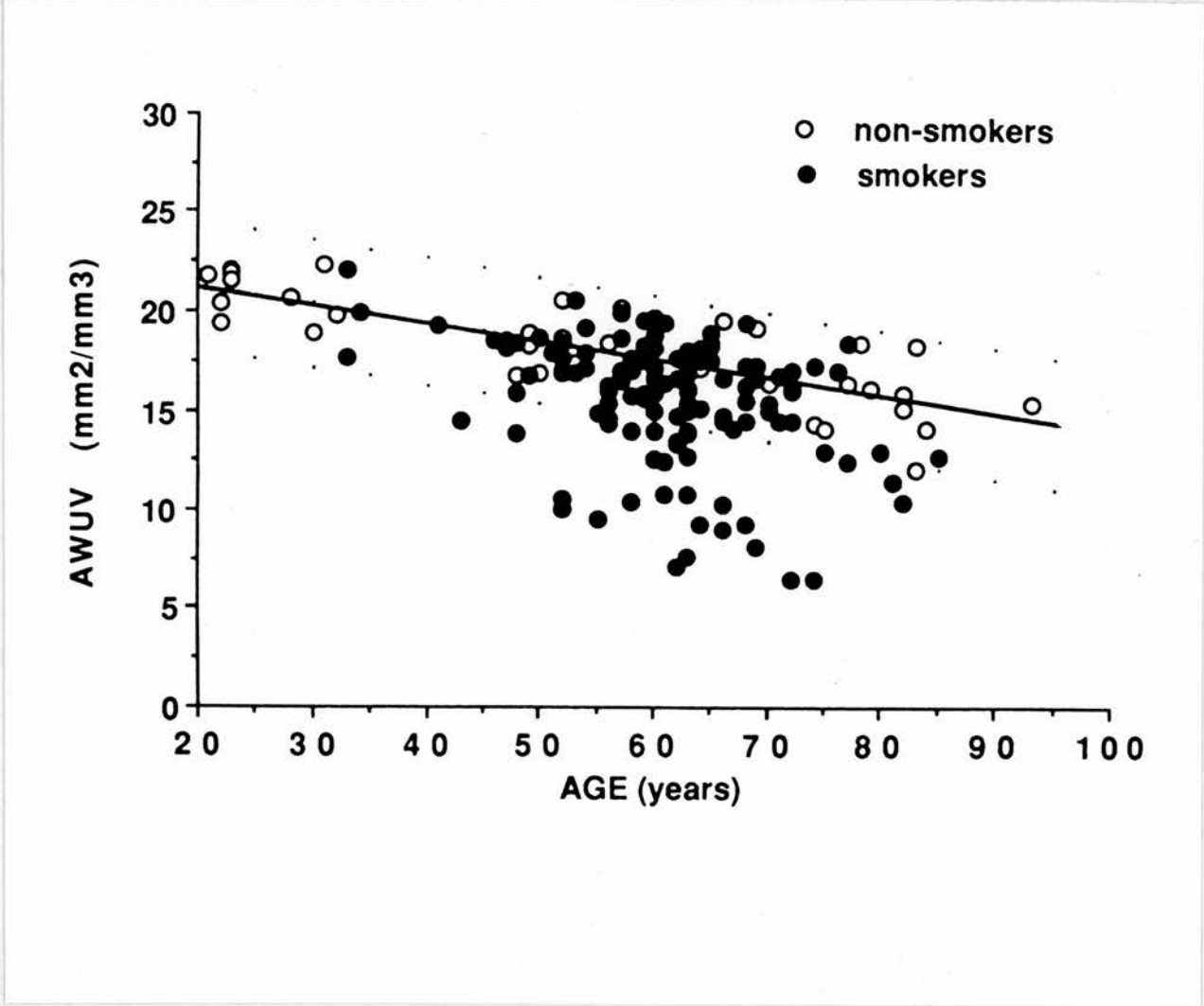


Figure 1.7 Graph showing alveolar wall surface area per unit volume of lung tissue (AWUV) of smokers and non-smokers plotted against age. A proportion of smokers have abnormally low AWUV values which do not show a dose dependant relationship.

Since oxidants can cause both cytotoxicity and genotoxicity, cigarette smoke exposure may lead to the development of lung cancer or emphysema, or both diseases in the same lung. Many studies have linked xenobiotic metabolising enzyme polymorphisms with susceptibility to lung cancer. Consequently, these enzymes may have a role in conferring susceptibility to emphysema, a disease also associated with cigarette smoke exposure. Many of the enzymes in the xenobiotic metabolising system are polymorphic, and disadvantageous polymorphisms can occur in relatively high levels in the general population. The xenobiotic metabolising system represents a very important first defence against tissue destruction. Deficiency in aspects of this defence resulting from polymorphism might therefore explain why there is not always a clear cut relationship between levels of cigarette smoke exposure and the subsequent presence and amount of disease.

1.2 Xenobiotic Metabolising Enzymes

Metabolism of compounds in the body can be divided into 2 different components, called Phase I and Phase II metabolism. Their function is the detoxification of both xenobiotics and internal compounds to make them more easily excreted from the body in the urine or bile (Gibson & Skett, 1986).

Although the majority of reactions carried out by the drug metabolising enzymes produce less reactive compounds, a minority of the products will be more reactive, resulting in a potential for toxicity in tissues (Gibson & Skett, 1986). The Phase I enzymes generally produce compounds which are suitable substrates for the Phase II enzymes. Phase II enzymes detoxify these substrates to produce non-toxic, excretable metabolites. As a result of this metabolism, particularly that of the Phase I enzymes, reactive, electrophilic molecules can be produced, which may attack membranes and DNA in peroxidation reactions. Procarcinogens can also be activated to their full carcinogenic forms by the Phase I enzymes in this way. Thus, although the drug metabolising enzymes function to protect the body from harmful chemicals, they can also act to produce metabolites which are more toxic than the original substrates.

1.2.1 Polymorphisms of xenobiotic metabolising enzymes

Polymorphisms exist in the genes of many of the major xenobiotic metabolising enzymes. From the above discussion of metabolism, it can be seen that where a polymorphism of one of these enzymes exists, it may have effects on the balance of reactive species and non-toxic metabolites circulating the body. Whether metabolism produces more reactive species, or more non-toxic metabolites depends on the enzyme involved, the site of action of the enzyme, and the substrate encountered by the enzyme.

An enzyme polymorphism is not necessarily significant in terms of disease pathogenesis because of the large degree of redundancy within the xenobiotic metabolising system. For example, a polymorphism which increased the activity of a Phase I metabolizing enyzme might produce an excess of highly reactive metabolites. Because of redundancy in Phase II metabolism, the excess of reactive species produced by the polymorphic Phase I enzyme may easily be detoxified before damage can occur. However, if there are coincident polymorphisms within Phase II enzymes, there may be a decrease in the protective capacity afforded by detoxification. The effects of the original polymorphism of Phase I metabolism might then result in toxicity.

1.2.2 Mechanisms of polymorphism production

There are many mechanisms by which polymorphisms of the drug metabolising enzymes can arise. These result in the production of altered protein, or no protein at

all, giving rise to the phenotypic changes seen. The mechanisms by which these arise include direct DNA mutations in the genes of the enzymes produced from deletion, insertion or rearrangement. Messenger RNA splicing may be affected to produce defective protein, and the stability of mRNA can also be altered, which will affect the amount of protein produced. At the protein level, a polymorphism may be seen where there is an alteration in the rate of synthesis of the protein or increased degradation of the protein. At the functional level, the enzyme may have an altered substrate affinity or substrate specificity and the rate of enzyme catalysis may also be affected.

Recently, gene amplification has been reported to occur in the gene of one polymorphic xenobiotic metabolising enzyme, cytochrome P4502D6 (Johanssen *et al*, 1993). Whether this phenomenon is specific to this single gene or not remains to be seen.

Examples of the above mechanisms include cytochrome P450 2D6, where mRNA splicing defects can result in at least 3 alternate mRNA transcripts (Meyer, 1990). Gene deletion, resulting in a lack of protein expression, is the mechanism whereby GSTM1 is polymorphic. Promoters and enhancers can affect metabolizing enzymes. An example of this is one of the polymorphic alleles of the cytochrome P4501A1 gene, which is thought to increase the induction of protein expression (Petersen *et al*, 1991).

1.2.3 Detection of xenobiotic metabolising enzyme polymorphisms

Many xenobiotic metabolizing enzymes are polymorphic. Historically, these polymorphisms have come to light because of phenotypic effects on metabolism of therapeutic drugs, for example the debrisoquine/sparteine polymorphism of cytochrome P4502D6. Polymorphisms were demonstrated to occur using biochemical methods which detected changes in protein conformation, for example gel-shift assays. With the advent of molecular biological techniques, many more polymorphisms are being identified through changes in DNA or RNA using restriction enzyme fragment length polymorphism (RFLP) analysis, single strand conformational polymorphism (SSCP) analysis, and direct cloning and sequencing of genes. Novel xenobiotic metabolizing enzymes are still being discovered, many of which may prove to have polymorphisms, once they have been characterized.

1.3 Xenobiotic metabolising enyzmes and susceptibility to disease

This study is concerned with polymorphic xenobiotic metabolizing enzymes and susceptibility to emphsema. In discussing which enzymes may be involved in the development of emphysema, emphasis will be made on enzymes which have been shown to be involved in other forms of disease. Several forms of xenobiotic metabolizing enzymes have been shown to be involved in susceptibility to cancer, and this has become the major focus of research into polymorphic xenobiotic metabolizing enzymes. Of importance in the development of tumours are the

enzyme families of the cytochrome P450s, the N-acetyl-transferases, and the glutathione S-transferases.

1.3.1 Cytochrome P450s

The cytochrome P450s are a gene superfamily of Phase I metabolizing enzymes whose function is the oxidative degradation of xenobiotics and the synthesis of steroids and bile acids. Many xenobiotics require metabolism to more hydrophilic forms before they can be excreted from the body in the urine or the bile (Gonzalez, 1992). It is thought that the high degree of polymorphism found in the cytochrome P450 superfamily may be due to the evolutionary development of new P450s through genetic change. Some changes may confer selective advantage on individuals, which results in the polymorphism becoming fixed in the population (Gonzalez, 1992).

The cellular location of the P450s is the endoplasmic reticulum or the mitochondrial membrane, where they function as the terminal oxidase in the electron transport chain (Gibson & Skett, 1986). P450s are divided into families by similarity of amino acid sequence. Mammals have 10 families of P450, which are then subdivided into various subfamilies and subsequent forms. The human genome is estimated to contain a minimum of 50 different P450 genes (Nebert *et al*, 1993). P450s from

different families share high sequence and amino acid homology (up to 40%) (Gonzalez, 1992). P450 genes demonstrate high degrees of conservation in the cytochrome P450 reductase binding site, the N-terminal membrane-bound domain, and the C-terminal haem-binding region (Smith *et al*, 1994).

Of the cytochrome P450s, the CYP1, CYP2 and CYP3 families are involved in xenobiotic metabolism (Gonzalez, 1992). A summary of the properties and expression of these xenobiotic metabolising enzymes is shown in Table 1.

CYP Gene Family	Number of genes in family	Substrate	Expression	Function
1A	2	Polycyclic Aromatic Hydrocarbons; Aromatic amines	Extrahepatic lung; Liver	Xenobiotic metabolism
2A	2 known	Aflatoxin B_1, Nitrosamines	Liver	Xenobiotic metabolism
2B	2 known	Aflatoxin B_1, Cyclophosphamides	Liver, lung, GIT	Xenobiotic metabolism
2C	5 known	Benzo[a]pyrenes	Liver, GIT	Xenobiotic metabolism
2D	3/4	NNK	Liver, brain	Xenobiotic metabolism

Table 1 Table summarizing the function, expression and substrates of the cytochrome P450 families of enzymes involved in xenobiotic metabolism (continued overleaf). GIT = gastrointestinal tract. NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

CYP Gene Family	Number of genes in family	Substrate	Expression	Function
2E	1	Ethanol, Nitrosamines, Benzene	Liver, brain, leucocytes	Xenobiotic metabolism
2F	1	3-Methylcholanthrene	Lung, liver	Xenobiotic metabolism
3A	4	Benzo[a]pyrenes, Aflatoxin B1, Cyclosporin	Liver, GIT, lung	Xenobiotic metabolism
4A	2-4	Fatty acids, Prostaglandins, Leukotrienes	Pancreas, liver	Xenobiotic and Endogenous metabolism
4B	1	Aflatoxin B_1, aromatic amines	Lung	Xenobiotic metabolism

Table 1 (continued) Table summarizing the function, expression and substrates of the cytochrome P450 families of enzymes involved in xenobiotic metabolism.

As indicated in Table 1, of the xenobiotic metabolising P450s, only enzymes from the 1A, 2B, 2F, and 4B families have been shown to be expressed in lung tissue. The CYP1A family comprises 2 genes, termed CYP1A1 and CYP1A2. Of the CYP1 enzymes, CYP1A1 metabolises polycyclic aromatic hydrocarbons, and is expressed in lung tissue, whereas CYP1A2 is primarily a hepatic enzyme, involved in nitrosamine and arylamine metabolism. The CYP2B subfamily contains one gene, CYP2B6, and an inactive pseudogene, CYP2B7P. The metabolic activation of cyclophosphamides and aflatoxin B_1 is mediated by CYP2B6. Cytochrome P4502F1 is expressed in lung at low levels, and has been shown to metabolise 3-Methylindole, an aromatic amine. CYP4B1 expression has been detected in lung tissue, and this enzyme metabolises aflatoxin B_1 and aromatic amines.

1.3.1.2 Cytochrome P450s important in lung disease

Of the cytochrome P450 enzymes expressed in lung tissue, CYP1A1, CYP2F1, and CYP4B1 all metabolise components of cigarette smoke (see Figure 1.8 for an example of this metabolism in the case of benzo[a]pyrene), and might therefore be involved in the mechanism of tissue damage which causes emphysema. Polymorphisms in the genes of these cytochrome P450s might alter their activity, thereby affecting the balance of cigarette smoke metabolites in the lung, and hence influencing susceptibility of the tissue to the development of damage resulting in emphysema. CYP4B1 is not known to be polymorphic.

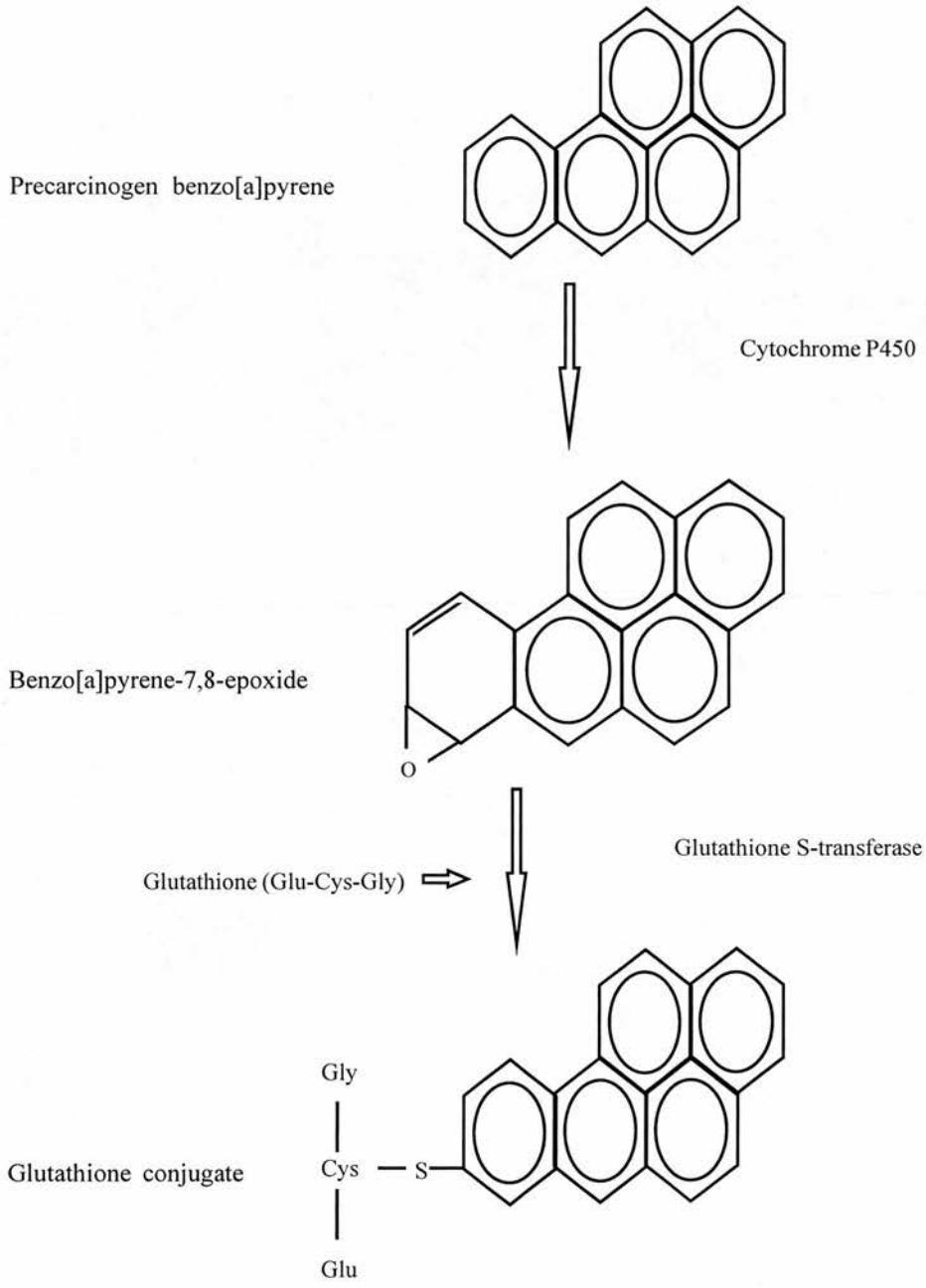


Figure 1.8 Diagram showing the detoxification pathway of benzo[a]pyrene by cytochrome P450s and Glutathione S-transferases. In an initial reaction, the cytochrome P450 activates the precarcinogenic benzo[a]pyrene to a derivative, benzo[a]pyrene-7,8-epoxide. The glutatione S-transferase then detoxifies the harmful derivative through its conjugation with glutathione. The glutathione conjugate undergoes further metabolism before being excreted in the urine or bile.

1.3.1.3 Cytochrome P4502F1

CYP2F1 has been shown to contain RFLPs in the 3' flanking region of the gene (Nhambuto *et al*, 1989), which were subject to Mendelian inheritance. A variant of CYP2F1, CYP2F1v, has been described, which contains 2 deletions (161bp at putative exon 4, 388bp at putative exons 8 and 9), and is thought to be non-functional due to the lack of a cysteine residue, which serves as the fifth thiolate ligand to the haem iron at the P450 site. CYP2F1 may be induced by cigarette smoke, since it has so far only been detected in the lungs of smokers, and in one liver sample from a smoker (Nhambuto *et al*, 1989). While this data suggests the possibility of polymorphism in the CYP2F1 gene, which may be of revelance to the metabolism of cigarette smoke, this preliminary work has not initiated further study into evidence of a role for this protein in disease susceptibility.

1.3.1.4 Cytochrome P4501A1

CYP1A1 has been localized to the chromosomal location 15q22-24 (Hildebrand *et al*, 1985). Several polymorphisms of CYP1A1 have been demonstrated (Bale *et al*, 1987; Spurr *et al*, 1987; Haugen *et al*, 1990; Hayashi *et al*, 1991a; Crofts *et al*, 1993). Of these polymorphisms, 2 have been shown to be involved in susceptibility to lung cancer. The first polymorphism is a *MspI* restriction enzyme fragment length polymorphism which is produced by a C-T mutation in the 3' non-coding region of the gene, 250bp downstream of the polyadenylation signal (Kawajiri *et al*, 1990).

The CYP1A1 MspI polymorphism has been shown to be related to ethoxyresorufin-O-deethylase (EROD) activity in peripheral lymphocytes (Landi *et al*, 1994). Individuals heterozygous or homozygous for the *MspI* polymorphism showed increased basal and inducible EROD activity. The CYP1A1 *MspI* RFLP has been shown to be associated with aryl hydrocarbon hydroxylase (AHH) activity (Petersen *et al*, 1991). High AHH inducibility has been shown to be associated with susceptibility to lung cancer (Kellermann *et al*, 1973a, 1973b; Kouri *et al*, 1982; Trell *et al*, 1985).

The *MspI* polymorphism is linked to a second polymorphism, an A to G transition in exon 7 of the gene, which results in an isoleucine to valine amino-acid substitution (Hayashi *et al*, 1991a; Hirvonen *et al*, 1992; Sivaraman *et al*, 1994). It has been demonstrated (Hayashi *et al*, 1991b, quoted in Kawajiri, *et al*, 1993) that when produced in yeast, the polymorphic valine allele of this gene results in an enzyme which exhibits a higher aryl hydrocarbon hydroxylase activity, and an increased mutagenic activity with benzo(a)pyrene in comparison with the isoleucine allele. It has been proposed that the cytochrome P450 1A1 exon 7 polymorphism produces a more highly inducible enzyme as a result of the valine amino acid substitution, and thus induction of gene expression by xenobiotics will be increased. If cytochrome P4501A1 metabolism functions to activate procarcinogens and produce more electrophilic and toxic metabolites, having an increased inducibility of the gene could be detrimental.

1.3.1.4.1 CYP1A1 is expressed in lung tissue

Antilla and colleagues, 1991, studied expression of CYP1 enzymes in lung using antibodies which detected both CYP1A1 and CYP1A2. Since CYP1A2 is expressed in the liver, lung detection of CYP1 enzymes should reflect CYP1A1 enzyme expression only. CYP1A enzyme expression was localized to the peripheral airways, in type I and type II alveolar epithelial cells, in ciliated columnar and cuboidal bronchiolar epithelium. CYP1A was present in the bronchial wall in minimal amounts, and where present, was localized in the epithelium of bronchial glands and the endothelium of capillaries. In 1994, in a study of smokers with lung cancer, Antilla and colleagues found an association between expression of CYP1A1 and susceptibility to bronchial tumours, which mainly consisted of squamous carcinomas.

1.3.1.4.2 CYP1A1 expression is induced by cigarette smoking

Cytochrome P450 1A1 expression can be induced by some of the xenobiotics which it functions to metabolize. In rodents, the CYP1A1 gene is regulated by an inducible enhancer termed the XRE (xenobiotic-responsive element) (Fujisawa-Sehara *et al*, 1986), and a promoter element which regulates the basic transcriptional activity (Yanagida *et al*, 1990), termed the BTE (basic transcription element). The XRE is situated upstream of the gene, while the BTE is adjacent to the TATA gene sequence. Both the XRE and the BTE sequences are required to induce expression of CYP1A1 (Kawajiri & Fujii-Kuriyama, 1991). The human CYP1A1 gene was found to contain homologous sequences to both the XRE and the BTE suggesting similar regulation

of gene expression (Kubota *et al*, 1991; Hines *et al*, 1988). The induction of CYP1A1 is mediated by the aryl hydrocarbon (Ah) receptor, a cytosolic receptor which is activated by polycyclic aromatic hydrocarbons, certain nitrosamines and some arylamines (Nebert & Jones, 1989).

CYP1A1 is thought to be one enzyme among several which mediates aryl hydrocarbon hydroxylase (AHH) activity. Levels of AHH activity have been correlated with the MspI CYP1A1 polymorphism (Petersen *et al*, 1991).

CYP1A1 is primarily extrahepatic, and has been shown to be predominantly expressed in lung and leukocytes. McLemore and colleagues showed by Northern blotting that cytochrome P450 1A1 is expressed in the lungs of cigarette smokers but not in the lungs of non-smokers (1990). Furthermore, a decrease in the amount of cytochrome P450 1A1 mRNA was demonstrated to be time-dependent in lungs of ex-smokers. This evidence strongly suggests that expression of this isoenzyme is induced by components of cigarette smoke. *In vitro* induction of cytochrome P450 1A1 has been shown using β-naphthoflavone, which induces CYP1A1 through the same receptor mechanism as polycyclic aromatic hydrocarbons (Andersson *et al*, 1993). In cancer patients, activity of CYP1A1 is increased in peripheral leukocytes as compared to leukocytes from healthy individuals (Rudiger *et al*, 1985; Bartsch *et al*, 1990). CYP1A1 activity has also been correlated with sister chromatid exchanges (van Poppel *et al*, 1992; Thompson *et al*, 1989). Two studies have found an association between AHH activity and DNA adduct levels (Bartsch *et al*, 1991;

Geneste *et al*, 1991). However, this result was disputed by Shields and colleagues in 1993, who could demonstrate no association between the CYP1A1 exon 7 polymorphism, which is thought to result in higher enzyme activity, and polycyclic aromatic hydrocarbon-DNA adducts in human lungs. It is possible that the study by Bartsch and colleagues measured non-PAH adducts, since the sensitivity of their method was not sufficient to identify the chemical nature of the adducts. A further study by Ichiba and colleagues (1994) found higher levels of aromatic DNA adducts in leukocyte DNA from individuals homozygous for absence of the CYP1A1 MspI polymorphism, as opposed to heterozygotes for the polymorphism.

1.3.1.4.3 Polymorphism of the CYP1A1 gene and disease susceptibility

Homozygosity for the CYP1A1 gene *MspI* RFLP has been shown to be more common in Japanese lung cancer patients (20.2%) than in normal controls (10.6%) (Kawajiri *et al*, 1990). No significant difference was seen between controls and patient groups with stomach, colon or breast cancer. Of the 4 main histological types of cancer, small and large cell carcinomas and squamous carcinoma are strongly associated with cigarette smoking, while adenocarcinoma is less strongly implicated (Kawajiri *et al*, 1993). When the lung cancer patient group was divided according to histological type of cancer, individuals homozygous for the *MspI* polymorphism comprised 26% of the individuals with squamous, small and large cell carcinomas, a highly significant difference from controls. The frequency of the CYP1A1 *MspI*

homozygous genotype was similar between controls and the group of adenocarcinoma patients.

Nakachi and colleagues, 1991, extended the study of CYP1A1 *MspI* polymorphism and susceptibility to lung cancer by genotyping squamous carcinomas and analysing the genotypes in terms of smoking history. Squamous carcinoma is the form of carcinoma with the clearest association with cigarette smoking (Lubin & Blot, 1984). Nakachi and colleagues demonstrated that individuals homozygous for the *MspI* polymorphism had a significantly increased risk for the development of squamous carcinoma at low dose exposure to cigarette smoke. At the lowest dose level of cigarette smoke exposure studied, which was 3×10^5 cigarettes (~40 pack years), the relative risk of developing squamous carcinoma in CYP1A1 MspI homozygotes was 7.3 (with risk of heterozygotes and homozygous non-polymorphic individuals as a base-line of 1.0). Heterozygotes and homozygous non-polymorphic individuals demonstrated a clear dose-dependent relationship between cigarette smoke exposure and relative risk of developing squamous carcinoma.

The results from Kawajiri and colleagues indicate that the CYP1A1 *MspI* RFLP is involved in susceptibility to lung cancer. Nakachi and colleagues (1991) have shown that this susceptibility is not directly dose dependant. Individuals homozygous for the *MspI* polymorphism are more susceptible to squamous carcinoma at lower levels of exposure.

The very significant susceptibility to lung cancer demonstrated by genotyping CYP1A1 in Japanese populations has not been found in Caucasian populations. This discrepancy may have arisen because Japanese populations have much higher frequencies of the CYP1A1 *MspI* polymorphism than have been found in Caucasian populations.

In 1991, Tefre and colleagues, failed to demonstrate an association between the *MspI* polymorphism and a population of Norwegian lung cancer patients. The proportion of the *MspI* rare CYP1A1 genotype was similar between 2 control groups from Norway and a Caucasian population from North America. The frequency of the CYP1A1 *MspI* allele was only one third as common in the Norwegian and North American populations as the polymorphism frequency in Japanese demonstrated by Kawajiri and colleagues, 1990. Shields and colleagues (1992) investigated whether or not there was association between the CYP1A1 *MspI* polymorphism and susceptibility to lung cancer in a US population. No statistically significant difference was demonstrated between lung cancer patients and case-matched controls. Hirvonen and colleagues, in 1992 and 1993, studied the CYP1A1 *MspI* polymorphism in the Finnish population, and found no association between the polymorphism and susceptibility to lung cancer.

Recently, Alexandrie and colleagues, 1994, failed to identify a statistically significant association between the CYP1A1 MspI and exon 7 polymorphisms and susceptibility to lung cancer. An increased frequency of the MspI polymorphism and

the valine substitution was observed in individuals with squamous cell carcinoma, but the difference from controls was not statistically significant.

Polymorphism of the CYP1A1 gene is thought to be correlated to the aryl hydrocarbon hydroxylase (AHH) inducibility phenotype (Petersen *et al*, 1991). AHH inducibility demonstrates a trimodal distribution in the population, which may indicate a genetic polymorphism in metabolism. Petersen and colleagues found that heterozygotes for the *MspI* polymorphism had higher AHH inducibility than predominant homozygotes, indicating a possible correlation between the CYP1A1 genotype and AHH phenotype. High AHH inducibility has been shown to be associated with susceptibility to lung cancer (Kellermann *et al*, 1973a, 1973b; Kouri *et al*, 1982; Trell *et al*, 1985).

CYP1A1 *MspI* polymorphism has also been shown to be associated with susceptibility to colorectal cancer in Japanese and Hawaiian populations, but not in Caucasian populations (Sivaraman *et al*, 1994). Although the MspI polymorphism was found to be in close linkage with the exon 7 polymorphism, no statistical association could be demonstrated between the exon 7 CYP1A1 polymorphism and colorectal cancer.

1.3.2 Glutathione S-Transferases

The glutathione S-transferases (GSTs) are a gene superfamily which detoxify xenobiotics by their conjugation with glutathione. In this manner, electrophilic compounds which might cause cytotoxic or carcinogenic damage may be removed from the body in the urine or bile. Glutathione S-transferases mediate Phase II metabolism, directly detoxifying xenobiotics and also detoxifying electrophilic metabolites derived from Phase I metabolism. Substrates for GST metabolism include aflatoxin B1, benzo[a]pyrene derivatives (see Figure 1.8), halogenated alkanes and alkenes, *trans*-stilbene oxide, and nitrosoureas (Smith *et al*, 1994). GSTs also function to transport hormones and hydrophobic compounds (Listowsky *et al*, 1988), and demonstrate glutathione peroxidase activity against lipids and DNA (Jakoby, 1978).

The expression of many GSTs is induced by the xenobiotics they metabolize, and GSTs are also regulated by hormones. Induction can be mediated by the binding of xenobiotics, antioxidants or glucocorticoids to responsive elements in the 5' flanking region of the GST genes (Rushmore & Pickett, 1993).

The GSTs have been classified into 7 classes, 6 of which are cytosolic, and at least 1 microsomal (Mannervik *et al*, 1992). The cytosolic classes are termed alpha, mu, pi, sigma and theta, and the letters A, M, P, S and T denote the class to which an enzyme belongs. Recently a novel GST class, termed kappa (GSTK) has been identified

(Pemble *et al*, 1996)Enzymes are assigned to a class based on their amino acid homology. GSTs have been shown to be expressed in most cells and tissues, but cellular expression depends on hormonal and environmental factors.

The mu and alpha classes of GSTs both encode at least 6 enzymes, the pi class encodes 2 enzymes, and the theta class has so far been shown to encode a single enzyme (Awasthi *et al*, 1994). Cytosolic GSTs form dimers, and the very high degree of sequence similarity between enzymes in each class of GST may result in heterodimerization occurring between different monomers. The pattern of expression of GSTs may therefore vary not only through cell and tissue specific induction, but also through variable heterodimerization of the monomers induced (Hussey *et al*, 1991).

Class alpha and pi GSTs have been implicated in resistance of tumours to chemotherapeutic agents (Hayes & Wolf, 1990).

Several studies of GST distribution in rats (Coursin *et al*, 1992) and mice (McLellan *et al*, 1992) have been published but there is little data on the distribution of GST isoenzymes in adult human lung (Awasthi *et al*, 1987; Fryer *et al*, 1986).

1.3.2.1 Polymorphic glutathione S-transferases

The mu class of GSTs contains a polymorphic enzyme, GSTM1. GSTM1 is known to metabolise cytotoxic components of cigarette smoke, including benzo[a]pyrene (Jernstrom *et al*, 1990). Substrates of GSTM1 include not only compounds of cigarette smoke which are directly cytotoxic, but also breakdown products of Phase I metabolism which may be cytotoxic (Gibson & Skett, 1986).

The GSTM1 polymorphism arises because of gene deletion and subsequent lack of protein expression, and affects approximately 50% of Caucasian populations (Seidegard & Pero, 1985; Seidegard *et al*, 1988). The exact nature of the deletion is unknown. Originally, the deletion was thought to involve a 267bp region including exons 4 and 5 of the gene. Recently, however, some evidence has been presented which suggests that the deletion may encompass the entire gene (Board *et al*, 1990; Seidegard *et al*, 1988). A number of studies have found that GSTM1 deletion is associated with an increased incidence of tumours. Tumours associated with GSTM1 deletion include adenocarcinoma of stomach and large bowel (Strange *et al*, 1991), pituitary adenomas (Fryer *et al*, 1993a), liver carcinoma (Harada *et al*, 1987), bladder cancer (Daly *et al*, 1993; Bell *et al*, 1993), multiple skin cancers (Heagerty *et al*, 1994), and colon cancer, particularly proximal colon cancer (Zhong *et al*, 1993a).

Several studies have also presented evidence contradicting some of the GSTM1 associations observed. Lin and colleagues, 1994, were unable to demonstrate

association between GSTM1 polymorphism and susceptibility to bladder cancer. Similarly, Zhong and colleagues (1993a) showed lack of association between the GSTM1 deletion and development of bladder cancer, or of breast cancer.

1.3.2.2 GSTM1 deletion and susceptibility to lung cancer

The evidence for an association of GSTM1 deletion with lung cancer in cigarette smokers is controversial. In 1986, Seidegard and colleagues presented the first evidence for an association between GSTM1 deletion and susceptibility to lung cancer, in a US population. Studies by Howie and colleagues (1990a) and Seidegard and colleagues (1990) confirmed this association. Furthermore, the results of Seidegard and colleagues (1990) indicated that in the US population, adenocarcinoma of the lung was particularly associated with the GSTM1 polymorphism.

Subsequently, Zhong and colleagues (1991) studying a UK population found that deletion of GSTM1 was not associated with lung cancer in total, but a significant correlation between the polymorphism and squamous carcinoma was demonstrated. Indeed, a negative correlation between GSTM1 deletion and adenocarcinoma was demonstrated. Hirvonen and colleagues (1993) also demonstrated a statistically significant association between GSTM1 deletion and susceptibility to squamous carcinoma in a Finnish population. In 1993, Brockmöller and colleagues, were unable to demonstrate any association between GSTM1 deletion and susceptibility to lung cancer in a German study group.

A study of Japanese individuals by Kihara and colleagues (1994) presented evidence which supports the role of GSTM1 deletion in susceptibility to squamous and small cell carcinomas of the lung. The association found by Kihara and colleagues was also seen to be related to cigarette smoke exposure, with higher frequencies of GSTM1 deletion seen in individuals with increased smoking history. Alexandrie and colleagues (1994) were similarly able to demonstrate an association between GSTM1 deletion and susceptibility to small cell carcinoma which was age-related. In patients under 61 years of age, the link between GSTM1 deletion and small cell carcinoma had an odds ratio of 3.1 (95% confidence intervals of 1.1-9.1) in comparison to a COPD group of controls, while patients aged less than 61 years had an odds ratio of 5.6 (95% confidence intervals of 1.4-22.5) compared to healthy controls. A very significant difference was observed between frequencies of the GSTM1 deletion in male and female sufferers of squamous cell carcinomas in the study by Alexandrie and colleagues. GSTM1 deletion was significantly associated with squamous carcinoma in women when compared to healthy controls (odds ratio 3.3, 95% confidence intervals of 1.2-9.7). Antilla and colleagues (1994) studied both GSTM1 and the CYP1A1 polymorphism discusssed in section 1.3.4.3. Antilla and colleagues demonstrated that in a smoking group of individuals with lung cancer, expression of CYP1A1 is associated with susceptibility to bronchial tumours ($p=0.001$). However, in individuals with CYP1A1 expression, presence of GSTM1 would seem to be protective against development of bronchial tumours ($p=0.037$).

1.3.2.3 GSTM1 deletion and susceptibility to non-neoplastic disease

The GSTM1 polymorphism has also been investigated in a number of non-neoplastic diseases. Davies and colleagues (1993) showed that the GSTM1 deletion is not involved in the development of primary biliary cirrhosis. Association between GSTM1 deletion and susceptibility to alcohol cirrhosis was investigated by Groppi and colleagues in 1991. No association was demonstrable between the GSTM1 polymorphism and development of alcohol cirrhosis. However, Harada and colleagues (1987), showed that the GSTM1 deletion is associated with susceptibility to chronic liver hepatitis and liver cancer.

1.3.2.4 GSTM1 involvement in specific cellular and biochemical processes

In 1991, Liu and colleagues, demonstrated a significant correlation between levels of aflatoxin B_1-DNA adducts and activity of GST mu enzymes (representative of the GSTM1 polymorphism, since individuals showed high or low conjugating activity). The results of the study by Liu and colleagues, indicate that GSTM1 may have a role in detoxification of aflatoxin B_1, and hence deletion of GSTM1 may increase susceptibility to liver cancer.

Deletion of GSTM1 has been shown to be associated with increased sister chromatid exchanges in lymphocytes of healthy smokers (van Poppel *et al*, 1992). The GSTM1 polymorphism has also been linked to levels of DNA adducts in lung tissue. Shields and colleagues (1993) observed a positive correlation between GSTM1 deletion and

DNA adduct levels. Ketterer and colleagues (1992) also presented preliminary results suggesting a negative correlation between GSTM1 and DNA adduct formation in lung tissue from smokers. In 1994, however, Ichiba and colleagues were unable to demonstrate any correlation between GSTM1 deletion and aromatic DNA-adduct levels.

Polymorphism of the GSTM1 and CYP1A1 genes have been shown to act synergistically in determining susceptbility to lung cancer in the Japanese population (Hayashi *et al*, 1992). Individuals with an intact GSTM1 gene and homozygosity for the non-polymorphic exon 7 allele of CYP1A1 were taken as a base-line of 1.0 for calculation of odds ratio. Individuals homozygous for the CYP1A1 exon 7 polymorphism and lacking GSTM1 were found to have odds ratios of 5.4 for lung cancers in total, 7.9 for squamous cell carcinomas and 3.8 for adenocarcinomas. In a Swedish population study, Alexandrie and colleagues, 1994, demonstrated no association between polymorphism of CYP1A1 and susceptibility to lung cancer, although a statistically significant age-related link between GSTM1 deletion and increased risk for small cell carcinoma was identified. When the lung cancer group was analysed for both genotypes, individuals polymorphic for CYP1A1 and the GSTM1 deletion had a significantly increased risk for developing squamous cell carcinoma (odds ratio of 3.0, 95% confidence intervals from 1.2-7.2). GSTM1 deletion has also been shown to be associated with increased risk for mutation in the tumour suppressor gene p53 in Norwegian lung cancer patients (Ryberg *et al*, 1994).

1.3.3 N-Acetyl Transferases

The N-acetyl transferases are Phase II metabolising enzymes which catalyse the acetylation of oxygen or nitrogen atoms using acetyl CoA as a cofactor. Substrates for N-acetyl transferases include aromatic amines, for example amino acid pyrolysates which are important dietary carcinogens formed during the frying or grilling of food, and drugs such as isoniazid, procainamide and hydralazine. Polymorphism of the N-acetyl transferases was first recognized through inter-individual variation in the metabolism of isoniazid, a drug used for treatment of tuberculosis. On the basis of their metabolism of isoniazid, individuals can be designated "fast" or "slow" acetylators. The slow acetylator trait is present in Caucasian populations at a frequency of 40-70%, but the frequency of the polymorphism is highly variable among different races (Smith, 1994). Genetic studies have identified 2 genes, NAT1 and NAT2, and a pseudogene, NATP at the chromosomal locus 8p 21.2-23.1. Both the NAT1 and NAT2 genes have been shown to be polymorphic as a result of point mutations within the genes (Blum *et al*, 1991; Hickman & Sim, 1991). Slow acetylation has been associated with increased risk for bladder cancer, presumably as a result of the decreased ability to detoxify aromatic amines, which are potent bladder carcinogens (Hein, 1988). The rapid acetylator genotype has been shown to be associated with increased susceptibility to colon cancer (Kadlubar, 1994). The mechanism by which this susceptibility results is

thought to be increased metabolic activation of procarcinogens by O-acetylation in the colon (Wolf, 1990).

While the N-acetyl transferases have been associated with susceptibility to both bladder cancer and colon cancer, there have been no reports of association with lung cancer. N-acetyl transferases are expressed in lung tissue, as well as in liver, colon, and red and white blood cells (Smith, 1994), and cigarette smoke contains aromatic amines, therefore, it may be speculated that polymorphisms of the NAT genes may affect lung disease.

1.3.4 Candidate polymorphic xenobiotic metabolising enzymes for investigating susceptibility to emphysema

While there may be a role for polymorphisms of N-acetyl-transferases and cytochrome P4502F1 in influencing disease susceptibility in the lung, there is not a great deal of evidence implicating these enzymes in lung disease. Other xenobiotic metabolising enzymes may be discounted for study since they are either not expressed in significant levels in lung or they do not metabolise components of cigarette smoke.

Of the polymorphic xenobiotic metabolising enzymes which have been discussed, the CYP1A1 and GSTM1 polymorphisms would seem to be most likely to be involved in increasing susceptibility to emphysema. In summary, both the GSTM1

and CYP1A1 gene polymorphisms have been linked to disease susceptibility, and in particular to lung cancer susceptibility. Expression of CYP1A1 is induced by cigarette smoke exposure (McLemore *et al*, 1990), and a functional difference in activity of the polymorphic variant of CYP1A1 has been identified (Hayashi *et al*, 1991b, quoted in Kawajiri, *et al*, 1993). CYP1A1 can activate polycyclic aromatic hydrocarbons (including benzo[a]pyrene and 3-methylcholanthrene), which are present in cigarette smoke, to produce highly reactive compounds (Kimura *et al*, 1986; Kawajiri *et al*, 1984). CYP1A1 might therefore be considered as an ideal candidate for investigation into the interindividual variation in susceptibility to emphysema. GSTM1 is known to detoxify components of cigarette smoke, including benzo[a]pyrene (Jernstrom *et al*, 1990). Expression of GSTM1 has not been clearly demonstrated in human lung tissue, but the enzyme is expressed in high levels in liver (Faulder *et al*, 1987). Even if GSTM1 is not expressed in lung tissue, the enzyme might have an important role in the development of emphysema, since panacinar emphysema may result from systemic damage (Saetta *et al*, 1994a).

AIMS

The aims of this thesis were to characterise the pattern and severity of emphysema in a series of biopsy and autopsy lung samples. This series of cases could then be used for genotyping studies into susceptibility to emphysema and lung cancer conferred by the polymorphic xenobiotic metabolising enzymes glutathione S-transferase M1 (GSTM1) and cytochrome P4501A1 (CYP1A1). To establish frequencies of the 2 polymorphisms in the Scottish population, a large group of anonymous blood samples was collected and genotyped, which comprised the control group for comparison in disease association. To investigate into whether or not the GSTM1 and CYP1A1 polymorphisms influenced chronic obstructive pulmonary disease (COPD), the clinical manifestation of emphysema, patients with COPD were also genotyped.

Previous expression of glutathione S-transferases in lung tissue had ascertained the expression of the classes mu, alpha, and pi by immunohistochemistry. However, the presence of GSTM1 in lung tissue had not been clearly demonstrated, and since the polymorphism may be involved in lung disease susceptibility, expression of this enzyme was further investigated using Western blotting and RT-PCR.

From the above studies of GST expression in human lung, and GSTM1 and CYP1A1 polymorphisms and susceptibility to lung disease, it was anticipated that information might be generated on the mechanisms by which lung diseases develop. If a role for polymorphic xenobiotic metabolising enzymes in influencing disease susceptibility was demonstrated, this would identify an important area for future research.

METHODS

2.1 Immunohistochemistry

Twenty-one lungs, or lobes of lungs obtained at pneumonectomy were studied. For histological preparation, lungs were fixed-inflated with buffered formalin for 24hr before selection of blocks from macroscopically normal or mildly emphysematous lung. Of the 21 lungs studied, all but 3 had peripheral tumours. These blocks were then processed to paraffin wax and sectioned at 2μ for immunohistochemical study.

Polyclonal rabbit antisera to human GST were the kind gift of Dr. JD Hayes (Harrison *et al*, 1989). Anti-GSTP was raised against purified human lung GSTP and the other antisera were raised against human liver preparations. Anti-GSTM was raised against liver GSTM which is the product of the GSTM1 gene. However since GSTM4 has greater than 90% sequence homology with GSTM1 (Zhong *et al*, 1993b) it is possible that the polyclonal antibody could react with the product of both genes. The immunostaining protocol used avidin-peroxidase and 3,3 diaminobenzidine detection (Harrison *et al*, 1989). Sections were preincubated with 50% pooled normal human serum in Tris buffered saline. This preincubation resulted in a marked reduction of background staining in controls using normal rabbit serum instead of polyclonal antibody. Antisera were used at a dilution of 1:200 in TBS containing 5% normal swine serum. Sections were assessed by two independent observers.

2.2 Western Blot Analysis of Bronchoalveolar Lavage Fluid

Four Bronchoalveolar Lavage (BAL) samples were obtained from healthy volunteers, and Western blotting of these BAL samples was carried out by Dr W Wallace. Following centrifugation to remove cellular debris, 10 ml aliquots were taken and placed in separate lengths of 14mm Viskose dialysis tubing (Medicell International Ltd., London). Protein was concentrated by dialysis against glycol methacrylate (BDH Chemicals, Poole) overnight at 4°C to give a final sample volume of 200µl. This was mixed with an equal volume of sample buffer (3.6mls distilled water, 1ml 0.5M Tris-HCl pH 6.8, 10% w/v SDS, 0.8ml 1M dithiothreitol, 0.8ml glycerol and 0.05ml 0.05% w/v bromophenol blue) and boiled for 5 minutes prior to loading on a 7.5% polyacrylamide gel. Protein was transferred to nitrocellulose and probed with GSTP antibody. Detection was by enhanced chemiluminescence (Amersham, UK).

2.3 Detection of human GSTM1 by Western blotting

Western blotting using antibodies to mu class GSTs was carried out on proteins derived from lymphocytes extracted from blood samples. If mu class GSTs were detected using this technique, Western blotting could then be applied to human lung tissue extracts to investigate mu class GST expression in the lung. If GSTM1 expression occurs at high levels in lung tissue, the difference in expression between individuals null for GSTM1 and those individuals with the intact gene, would

provide evidence that where the gene is not deleted, GSTM1 expression occurs in lung tissue.

2.4 Selection of samples for study of disease susceptibility and collection of control population samples

2.4.1 Collection and assessment of lung samples with emphysema

The lungs studied in this thesis were selected from biopsy and autopsy cases which entered the Pathology Department of Edinburgh University as part of the routine biopsy service provided by the department.

The majority of lung biopsies received were suspect lung cancer cases as diagnosed by chest x-ray, and biopsy tissue generally comprised the lung lobe, or lobes, in which the abnormality arose. Lung samples received by the department were cut into slices, and blocks of 2cm square were cut for processing in paraffin wax to enable the production of microscopic slide specimens. Lung tissue selected for the production of slides usually included several blocks from areas affected by disease, areas unaffected by disease, and in lung where tumour was found, samples from the lymph nodes, in particular the hilar lymph node. The remaining lung tissue was fixed and stored in formaldehyde. Autopsy lung samples were stored in a similar fashion if the tissue was shown to have lung disease following examination.

Macroscopic emphysema is defined as airspaces measuring 1mm or greater in diameter. All lung samples stored in formaldehyde were examined for the presence, type and extent of macroscopic emphysema. This thesis concerns centriacinar and panacinar emphysema only, and lung samples were assessed for the presence of these 2 types of emphysema alone. The extent of the lung affected by emphysema was estimated in each case: for centriacinar emphysema, the approximate number of lesions visible on the cut surface of the lung was recorded; for panacinar emphysema, the percentage involvement of the lung was calculated. Often, although airspaces were not as large as 1mm in diameter, emphysema was visible since the cut surface of the lung appeared grainy, and less smooth than unaffected lung tissue. Areas of the lung affected in this manner were described as Grade 1/2 panacinar emphysema, and the percentage of the lung involved was recorded.

Assessment of the extent of emphysema was recorded as described above, but to give some idea of the severity which was encountered, samples were classed as having mild, moderate or severe emphysema. The lung biopsy and autopsy cases assessed were subject to considerable variation, for example, the size of samples varies depending on which side of the lung the lobe is removed from, and how far into the middle of the lobe the tissue is cut. Although several lung slices were assessed for each case, assigning severity is both inaccurate and arbitrary. However it is useful to summarize the extent of disease severity found in the cases assessed.

Mild emphysema was classified as a lung surface containing 20% or less involvement of panacinar emphysema, or 20 or less lesions of centriacinar emphysema. Between 21 and 50% involvement of the lung with panacinar emphysema, and tissue containing 21-50 centriacinar lesions, was classed as having moderate disease. Panacinar emphysema affecting over 50% of the surface of the lung, and centriacinar lesions numbering over 50, were classed as severe emphysema. Where both panacinar and centriacinar emphysema were present, severity was always assessed with reference to the more severe pattern. While this classification of severity is useful, it is purely based on assessment by eye, and will not correlate to the extent of clinical symptoms of the patients. Furthermore, the percentage involvement of the lung tissue by panacinar emphysema is variable since the severity of damage will vary, and in centriacinar emphysema, lesions may vary in size. Age must also be considered in the assessment of panacinar emphysema. As individuals age, the airspaces in their lungs increase in size (Gillooly & Lamb, 1993a), therefore what might be classified panacinar emphysema in a younger person's lung tissue might represent normal tissue of an older individual.

Macroscopic emphysema is defined as airspaces in the lung of 1mm diameter or greater. Since the average diameter of an airspace measures 250μm (Schreider *et al*, 1981), by the time sufficient damage has occurred for it to be visible macroscopically, three quarters of the airspace wall must have been destroyed. It is therefore important to be able to detect and measure damage which, although not

macroscopically visible, is occurring in the lung. This early damage is termed microscopic emphysema.

Microscopic emphysema was measured using histological specimens of lung tissue by Dr Gillooly. Six blocks of lung tissue were cut from each lobe, and were analysed using the fast interval processor, an automated photosensitive scanning device which counts the number of intercepts of alveolar walls with a test line (Gillooly *et al*, 1991). From the total number of intercepts counted, the mean linear intercept (Lm) can be calculated (Dunnill, 1987). The Lm value obtained may then be used to calculate the alveolar wall surface area per unit volume of lung tissue (AWUV) with the equation AWUV = 2/Lm (Gillooly *et al*, 1991).

With increasing age, the number of alveolar walls decreases (Gillooly & Lamb, 1993a), hence in order to determine the presence of microscopic emphysema, AWUV values must be compared to the limits of normality of the general population. The distribution of AWUV values in a non-smoking population were determined by morphometry (Gillooly & Lamb, 1993b). The lower limits of AWUV normality (95% limits) in non-smokers can be seen in Table 2. Where the AWUV value obtained from a lung falls below the 95% limits of normality in the general population, this individual can be classed as having microscopic emphysema.

Information on patients from whom the lung tissue had been obtained was collected by Dr Gillooly and myself, and included details of disease, sex, age, smoking history,

and lung function test results. Since this data was obtained from biopsy reports and clinical reports, the information collected was not always complete for each patient.

Age (years)	AWUV (mm^2/mm^3)
20	17.99
25	17.56
30	17.12
35	16.68
40	16.24
45	15.79
50	15.33
55	14.87
60	14.41
65	13.94
70	13.47
75	13.00
80	12.52
85	12.03
90	11.54

Table 2 - Table of the lower limits (95% limits) of alveolar wall surface area per unit volume of lung tissue (AWUV) in the non-smoking population (Gillooly & Lamb, 1993b).

2.4.2 Selection of paraffin wax embedded lung samples for analysis

Selection of blocks of archival tissue for PCR analysis was carried out on the basis that tissue yielding as much DNA as possible was required. It was anticipated that poor DNA yield generated from blocks containing peripheral lung might represent a problem for PCR of these samples. Hence, blocks containing bronchi and/or lymph nodes were favoured over those with only peripheral lung. The expected yield from these blocks would be far greater since the cell volume in peripheral lung will be much lower than that seen in bronchial lung or lymph node tissue. Furthermore, lung tissue, particularly that of individuals biopsied for lung cancer, is likely to contain tar and other chemicals from cigarette smoke exposure which may inhibit either the DNA extraction or PCR amplification from this tissue. The presence of tumour was avoided, although selection was made on the basis of eye examination, therefore tumour tissue presence cannot be completely ruled out. It is well characterised that tumour cells may contain mutations of their DNA, hence genotyping of tumour tissue may give results which do not reflect the genotype of genomic DNA. By avoiding tumour in the blocks selected, it was expected that the prevailing DNA yieded for PCR analysis would be that of normal tissue. If areas of tumour were present in the only blocks available for analysis, wherever possible, these were removed from the adjacent areas of normal tissue using a scalpel and discarded.

2.4.3 Blood samples

Two sets of blood sample groups were collected for this project. The first, a control group, comprised 400 blood samples collected by Dr P-L Yap of the Edinburgh and South-East Lothian Blood Transfusion Service. These samples were completely anonymous, thereby providing a control group for comparison with disease cases in a cross-sectional study of disease susceptibility. The second set of blood samples were collected from patients attending a chronic obstructive airways disease clinic run by Dr W Macnee, Department of Respiratory Medicine, Royal Infirmary of Edinburgh. Clinical details including age, sex, smoking history and measurements of disease severity were collected for all cases.

2.4.4 Fresh lung tissue

Fresh lung tissue was only infrequently used in this study, to provide high quality DNA for optomisation of the GSTM1 and CYP1A1 PCR protocols. The tissue was obtained from normal tissue in lung resections for cancer.

2.5 DNA extraction

Fresh tissue samples were dissected into cubes of approximately $0.5cm^3$, DNA was extracted using the method of Jackson and colleagues (1990) , as described for paraffin wax-embedded samples. The remaining fresh lung tissue was snap frozen in liquid nitrogen and stored at -70°C. DNA was extracted from blood samples using the method of Smith and colleagues (1992).

Two sections of 10μm thickness were cut from paraffin wax embedded tissue blocks using a microtome. DNA was extracted from paraffin wax-embedded tissue by incubating sections with 400 μl buffer A (50mM KCl, 2.5mM $MgCl_2$, 20mM Tris-HCl, pH 8.0, 0.45% Nonidet P-40, 0.45% Tween 20), containing 200 mg/ml Proteinase K at 55°C for 2 hours. After boiling for 20 minutes, samples were stored at -20°C (Smith *et al*, 1992). Following brief centrifugation, 15 μl of the supernatant was used to amplify DNA fragments by PCR.

The amount of paraffin wax-embedded material was limited to 2 10μm sections for extraction since it is well known that inhibitors of PCR are present in paraffin wax, and in the fixation buffers used in tissue preservation and embedding. The amount of paraffin embedded material must be sufficiently large for DNA to be extracted whilst keeping inhibitory factors as limited as possible. The use of more numerous sections was investigated, but no increase in PCR yield was observed.

The use of xylene in deparaffinisation of lung tissue sections was investigated, as was phenol:chloroform washing and ethanol precipitation of the extracted DNA following Proteinase K incubation, but PCR yield was not significantly increased with the addition of these extra steps. The efficacy of the chelating agent Chelex^{R} 100 (Biorad, Richmond CA) in increasing PCR yield was also investigated following the report of Stein & Raoult (1992). However, no increased yield was demonstrable using this reagent.

2.6 Polymerase Chain Reaction

2.6.1 Reverse Transcription Analysis

To determine the expression of GSTM1 and GSTM4 in lung, RNA was extracted from 3 lung tissue samples. Of these samples, 2 were disease-free, while the third sample was derived from lung tissue containing a pneumococcal infection. RNA was extracted from fresh lung tissue using RNAzol B (Biogenesis, UK) as per manufacturers instructions. cDNA was then amplified using oligodT (Gibco BRL, UK), with a standard mix of PCR reagents, Superscript enzyme and dithiothreitol (Gibco BRL, UK), and RNAsin (Promega, UK). Reverse transcription was carried out at 37^{o}C for 10 mins, 42^{o}C for 1 hour, 50^{o}C for 10 mins and 94^{o}C for 10 mins. Primers were chosen which amplified both GSTM1 and GSTM4 to produce cDNAs of 657bp in length which were distinguished by digestion with *NcoI* restriction enzyme (Gibco BRL, UK). GSTM1 gave expected fragments of 311, 221 and 125bp,

whereas GSTM4 produced fragments of 436 and 221bp. Therefore four fragments of 436,311,221 and 125bp were expected if tissue expressed both genes. Digestion took place overnight at 37°C, before analysis by agarose gel electrophoresis (FMC Bioproducts, US). DNA was visualised by ethidium bromide staining and examination under ultraviolet irradiation.

The primers (Oswell DNA Services, UK) used were:

P1: 5'-ATGCCCATGATACTGGGGTACTGG-3'

P2: 5'-CTACTTGTTGCCCCATACATCCAT-3'

2.6.2 GSTM1 PCR assay

The PCR buffer was a standard mix of nucleotides and contained 3% DMSO (Sigma, US). The amplification was achieved by adding 5U of Taq polymerase (Promega, UK) in a hot start, and undergoing 35 amplifications of 59°C for 30 seconds, 72°C for 90 seconds, and 94°C for 30 seconds. Amplified DNA was electrophoresed in 3% agarose gels (FMC Bioproducts, US). DNA was visualised by ethidium bromide staining and examination under ultraviolet irradiation.

The primer strategy used was a modification of that used by Zhong and colleagues (1993b) and Shea and colleagues (1990) (Figure 2.1). This method allowed the amplification of both GSTM1 and GSTM4 and hence provided a positive control for each reaction, since GSTM4, which is not known to be polymorphic (Zhong *et al*, 1993b), was always amplified. This was particularly important as DNA extracted

from archival paraffin blocks may be of poor yield. Hence a loss of GSTM1 identified in this way could be distinguished from a failed PCR reaction.

The following oligonucleotide primers (Oswell DNA Services, UK) were used in the PCR reaction:

22y: 5'-CTGCCCTACTTGATTGATGG-3'

23y: 5'-ATCTTCTCCTCTTCTGTCTC-3'

24y: 5'-TTCTGGATTGTAGCAGATCA-3'

The primers 22y and 23y when used together in a PCR reaction amplify a DNA fragment of 202bp in length, while the use of 22y and 24y together results in the amplification of a fragment of 275bp long.

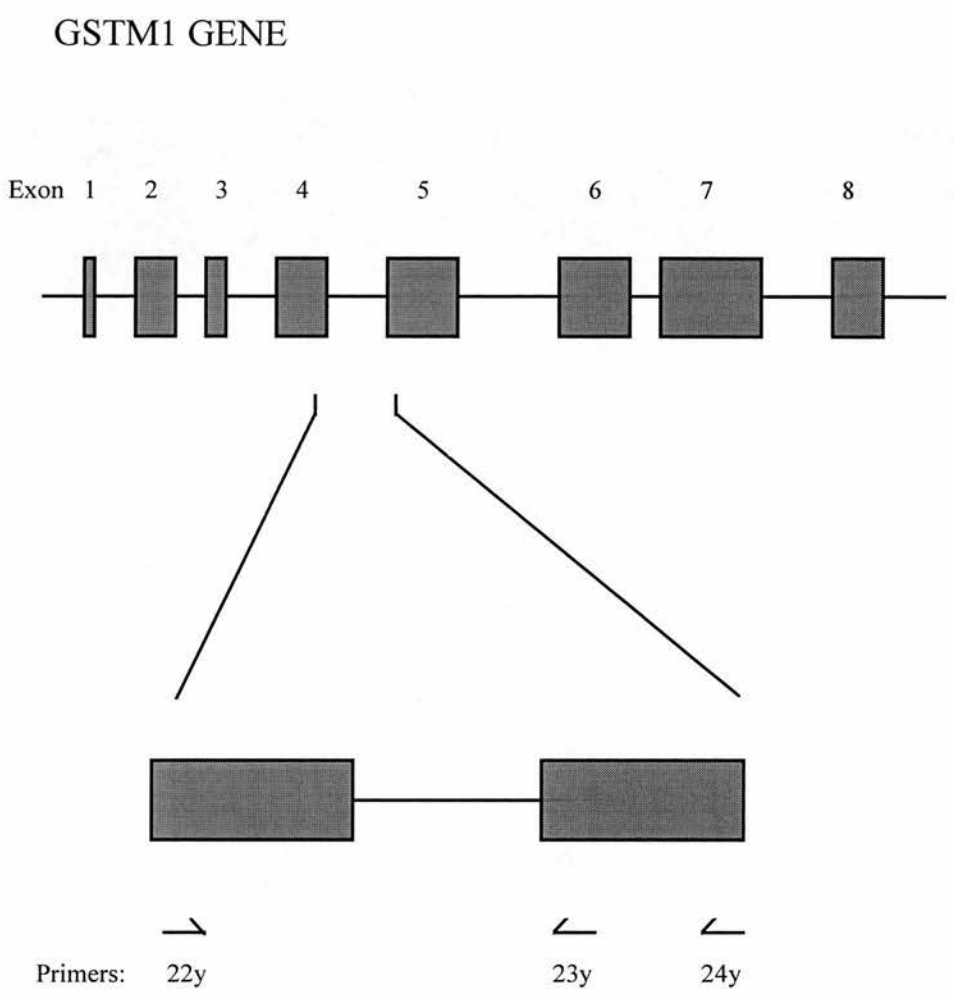


Figure 2.1 Schematic diagram of the GSTM1 gene. The upstream primer (22y) is situated in exon 4, whereas both downstream primers (23y and 24y) are located in exon 5. Both 22y and 23y anneal to GSTM4 in addition to GSTM1, and thus serve as a positive control for the PCR assay. Primer 24y is specific for GSTM1.

2.6.3 CYP1A1 PCR Analysis

Genotyping was carried out by polymerase chain reaction analysis using buffer and 1.5mM $MgCl_2$ (Promega, UK), 150μM deoxynucleotides (Pharmacia, UK), 5% DMSO (Sigma, US), 25 pmoles of primer (Oswell DNA Services, UK) and 2.5 units of Taq polymerase (Promega, UK). The primers used were from intron 6 and exon 7 and the sequences were:

upstream 5'-AAAGGCTGGGTCCACCCTCT-3';

downstream 5'-AAAGACCTCCCAGCGGGCCA-3'.

The downstream primer incorporates a mismatched base to engineer an *Nco*I restriction enzyme site in the PCR products derived from the Ile^{462} allele of the gene. In the Val^{462} allele of the gene, this restriction site is lost. The primers amplify both alleles, and the genotypes are distinguished by *Nco*I digestion of the products. An *Nco*I restriction enzyme site located upstream of the mutation in either genotype serves as a positive control for PCR product digestion (Figure 2.2). PCR products were electrophoresed in 3% NuSeive and SeaKem agarose (FMC Bioproducts, US), and restriction enzyme digestion fragments were electrophoresed in 3% Metaphor agarose (FMC Bioproducts, US).

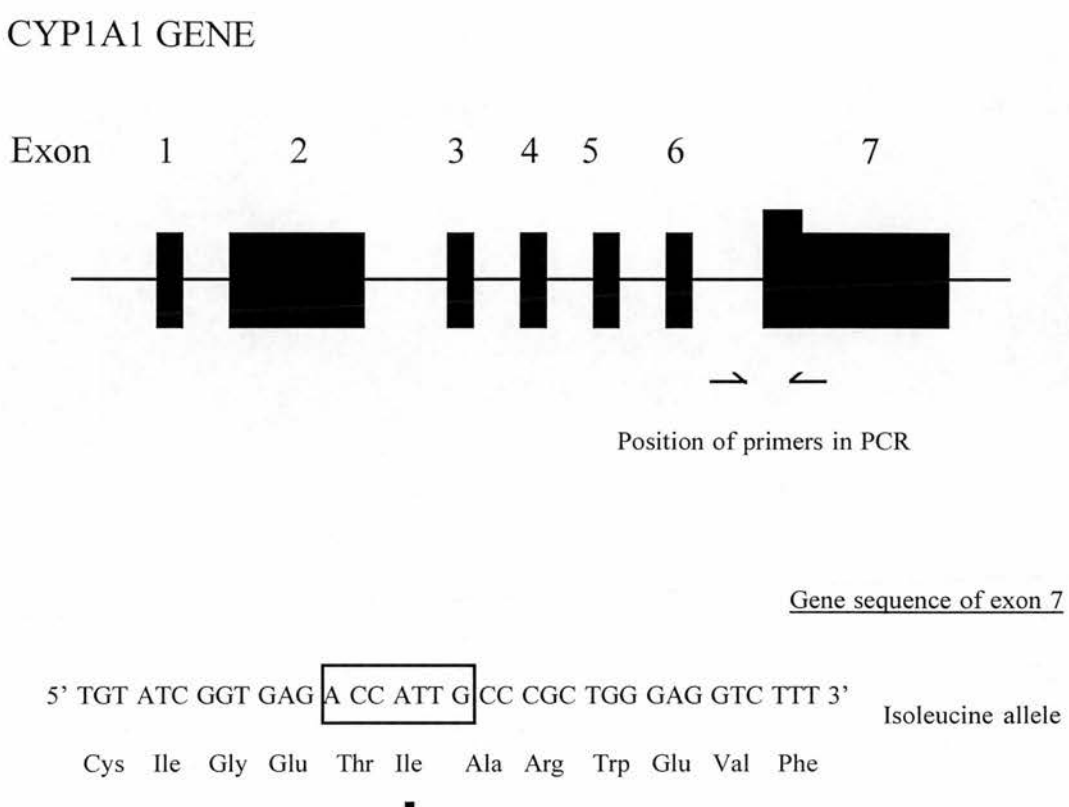


Figure 2.2 Schematic diagram of the CYP1A1 gene structure showing the PCR assay and restriction enzyme analysis used in genotyping. * indicates the position of the base change (T to G) introduced by the downstream primer to create a *NcoI* restriction enzyme recognition site (box). The substitution of a G for an A in the valine allele of the gene (as indicated by an arrow) destroys this *NcoI* enzyme recognition site.

2.64 Statistical Analysis

Stastical analysis of the genotyping results for both the GSTM1 and CYP1A1 polymorphisms was carried out using the Minitab for Windows 6.0 Statistics Package for $\chi 2$ analysis, and the Instat Statistics Package (GraphPad Software, Instat V2.05a) for Fishers exact test calculations. All statistics were calculated from a null hypothesis for an unmatched cross-sectional study of disease association (Campbell & Machin, 1993).

2.7 Sequencing of the CYP1A1 gene

The PCR fragment amplified from the CYP1A1 gene using the primers described was sequenced to check that the mismatch introduced by the 3' primer did not affect the remainder of the amplimer in any way. Initially, sequencing was carried out by cycle sequencing the fragment using the 3' primer. When this method was unsuccessful, cycle sequencing using the 5' primer was carried out, but this method failed to provide readable sequence. The CYP1A1 PCR fragment was then cloned and sequenced by direct sequencing.

2.7.1 Cycle sequencing of the CYP1A1 gene

Cycle sequencing was carried out using the Stratagene Cyclist DNA Sequencing Kit (Stratagene, UK). A master mix containing 200 fmol template, 1 pmol primer, sequencing buffer, 10 μCi ^{35}S radioactive label, 1 μl Exo$^-$*Pfu* DNA polymerase, 4 μl DMSO was made up to 30 μl volume with water, and 7 μl of the mix added to 3 μl aliquots of the 4 dideoxyribonucleotides. Samples were mixed and overlaid with 30μl paraffin oil before cycling in a thermocycler. Cycling was carried out at an initial 5 minute denaturation temperature of 95°C, followed by 30 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. To stop the cycling reaction, 5 μl of stop solution was added to the samples, and the reactions were heat denatured for 5 minutes at 80°C before loading onto a sequencing gel.

2.7.2 Cloning of the CYP1A1 PCR Product

PCR products were purified using Glassmax columns (Gibco BRL, UK), before their ligation into the PGEM-T vector (Promega, UK). Ligation reactions were transformed into JM109 competent cells (Gibco BRL, UK), which were grown on L-amp plates treated with 100µl 2% X-Gal and 100µl 40mM IPTG to allow blue/white selection of transformed cells to occur. Transformed colonies containing vector were selected and grown in L-broth containing ampicillin, and the vector was isolated using the Wizard Miniprep System (Promega, UK). Miniprep isolated DNA was digested with *NcoI* restriction enzyme (Gibco BRL, UK) at 37°C overnight to identify vectors containing CYP1A1 DNA. Colonies with vectors containing CYP1A1 inserts were then grown in 100ml L-broth containing Ampicillin for preparation of DNA by maxiprep using the Qiagen-tip system (Qiagen, UK). DNA recovered by maxiprep was quantified on ethidium bromide agar plates, before the application of chain termination sequencing.

2.7.3 Chain termination sequencing of cloned CYP1A1 PCR products

The double stranded vector containing the CYP1A1 PCR product (5µg concentration) was denatured with 2M sodium hydroxide, 2mM EDTA, neutralized with 2M ammonium acetate and precipitated with ice cold absolute ethanol. Following centrifugation, the DNA pellet was washed with 70% ethanol, before drying under vacuum and resuspension in water. Sequencing was then carried out using the USB Sequenase kit (Amersham, UK). Sequencing gels were visualized using autoradiograph film (Kodak, UK).

2.8 Novel Thr-Asn CYP1A1 variant detection PCR

The PCR assay used to genotype the CYP1A1 Ile-Val^{462} polymorphism could not differentiate the Thr-Asn variants. A PCR assay was therefore designed to differentiate the Thr-Asn variants in order to investigate the frequency of this sequence in the general population. By introducing a base change into the upstream primer, a *Sau96I* restriction enzyme site could be engineered into PCR products. This restriction enzyme recognizes the threonine variant of the CYP1A1 gene, but will not cut the asparagine variant.

2.8.1 Genotyping of individuals for the novel CYP1A1 polymorphism

Twenty-seven individuals from the Scottish control group previously identified as heterozygotes for the Ile-Val^{462} CYP1A1 polymorphism were genotyped for the novel polymorphism by PCR and *Sau96I* restriction enzyme analysis (Gibco BRL, UK). Using the previous *NcoI* restriction analysis, distinction between the Ile-Val462 and the Thr-Asn461 polymorphisms was not possible since both mutations destroy the *NcoI* site. With the introduction of a base change in the primer, a restriction enzyme site can be engineered, which is destroyed by the novel CYP1A1 sequence. The assay employed can be seen in schematic form in Figure 2.3. The primers (Oswell DNA Services, UK) used were:

upstream primer: 5'-AGCGGAAGTGTATCGGTGGG-3'

downstream primer: 5' -GAAGCATGCCTGCTGTGAGC-3'

Following DNA restriction, fragments were electrophoresed on 6% polyacrylamide gels with DNA molecular weight marker ϕX174 (Gibco BRL, UK).

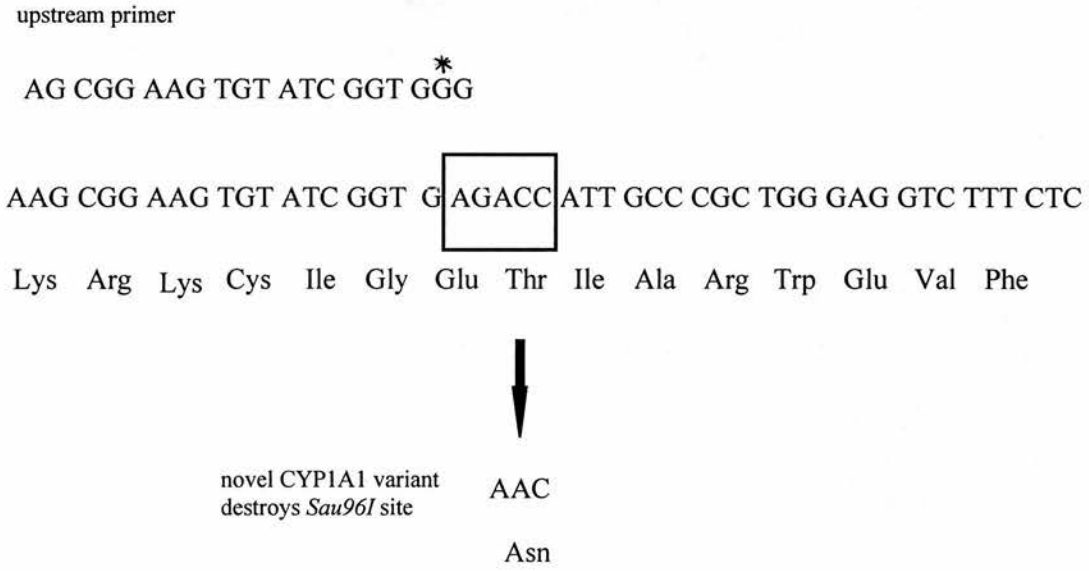
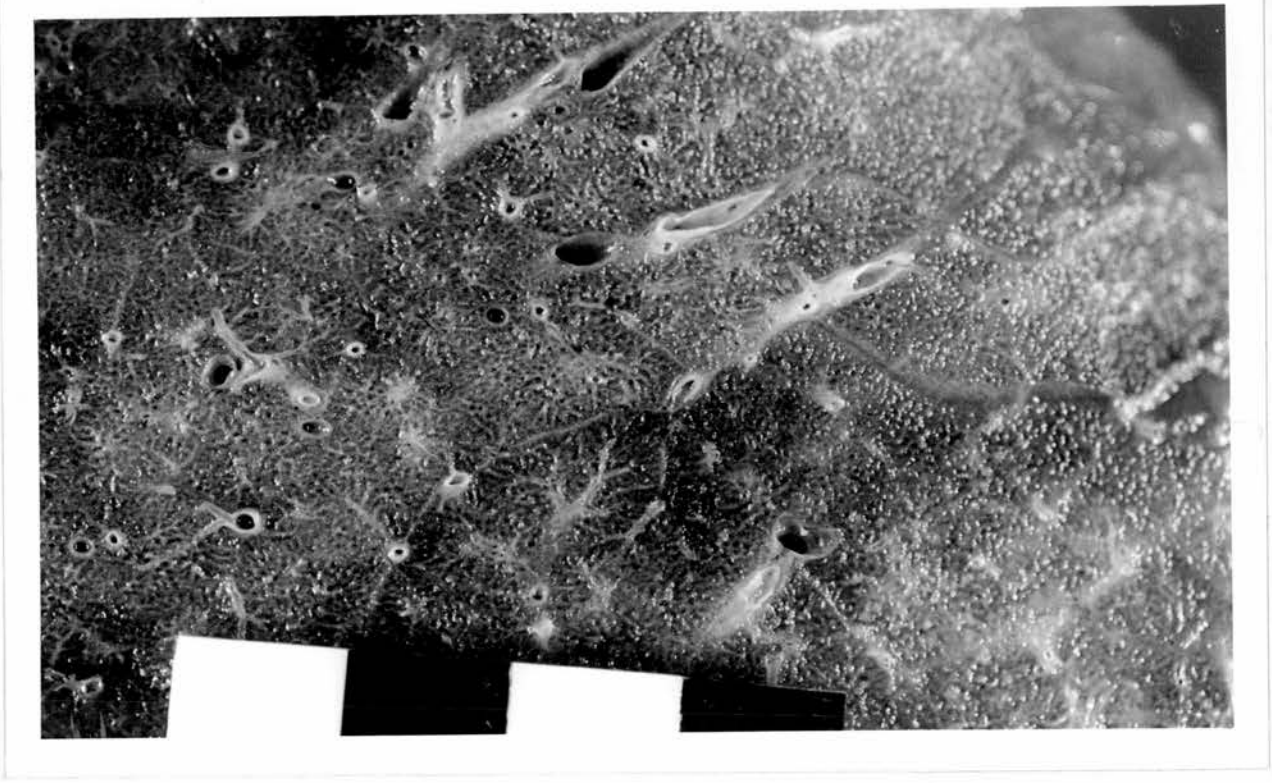


Figure 2.3 Schematic diagram of the PCR assay and restriction enzyme analysis used to genotype for the novel Thr-Asn^{461} polymorphism of the CYP1A1 gene. The base change introduced to the PCR fragment using the upstream primer is indicated by a *, while the *Sau96I* restriction site is indicated by a box.

3.1 Assessment of emphysema in lung samples

In all cases the extent of emphysema was of mild to moderate degree which was expected since all cases had been obtained from patients deemed fit for surgery, who had FEV_1 of at least 1.4 l/s. No difference in gender, age or tumour type was found between subgroups with different forms of emphysema.

The distribution of emphysema patterns and severity is summarised in Table 3.1. This study is concerned primarily with centriacinar and panacinar emphysema patterns. Severity was assessed by eye on the cut surface of the lobe of the lung which had been removed for the biopsy. Figures 3.1-3.5 show examples of the varying emphysema patterns and severity encountered in the course of this study.

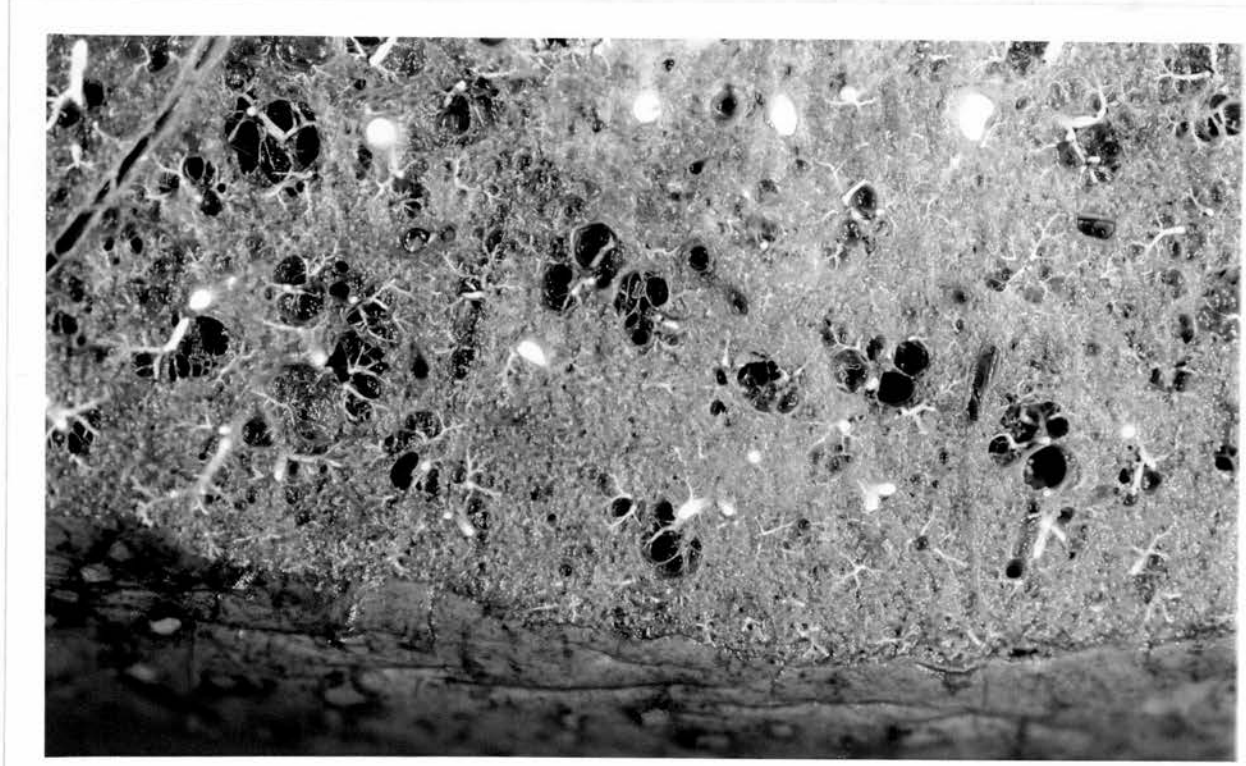


Scale bar: 1cm

Figure 3.1 Normal lung with no evidence of emphysema

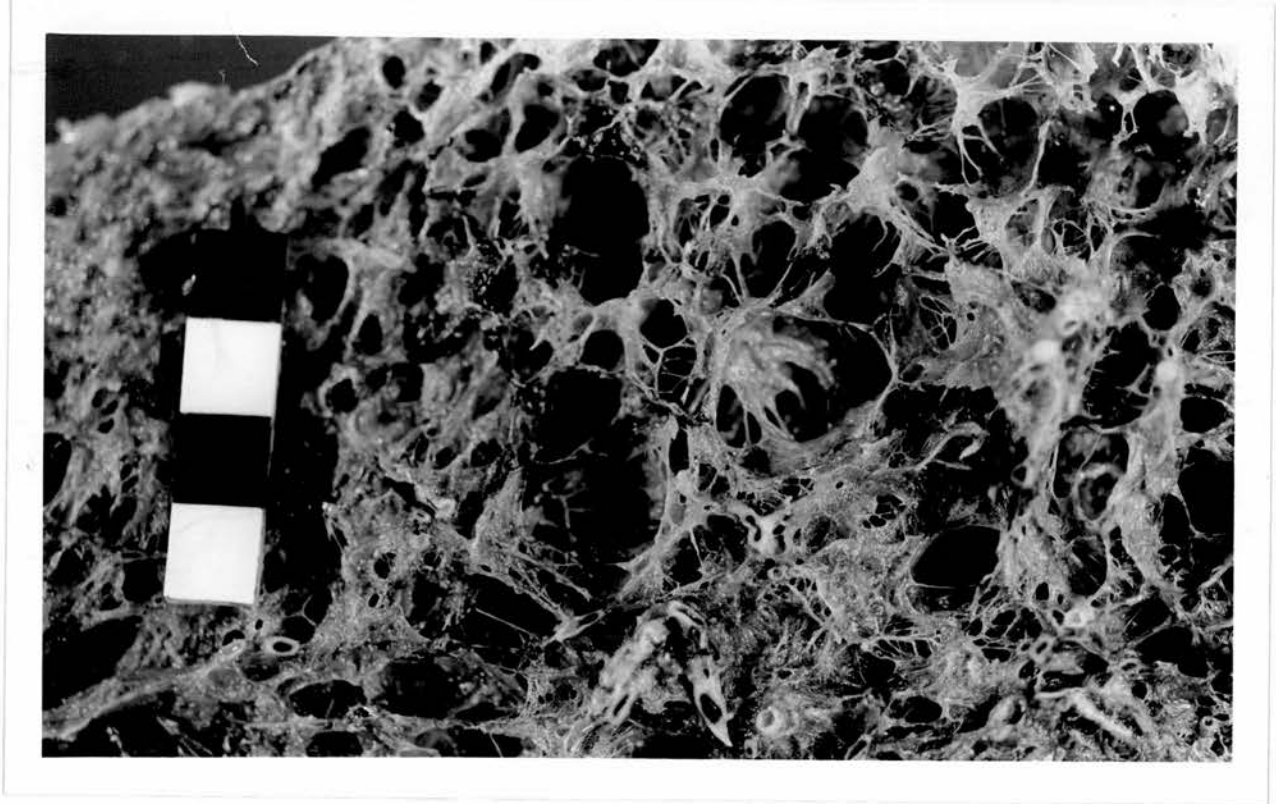
Scale bar: 1cm

Figure 3.2 A lung sample with mild panacinar emphysema demonstrating the uniform distribution of this type of emphysema. Although the airspace size is not very great, a significant difference can be seen in this sample when compared to the normal lung (Figure 3.1). It may appear from this photograph that panacinar emphysema has a tendancy to be peripheral, however this is a consequence of the lack of lung inflation which occurs when the sample is removed from formalin for photography.



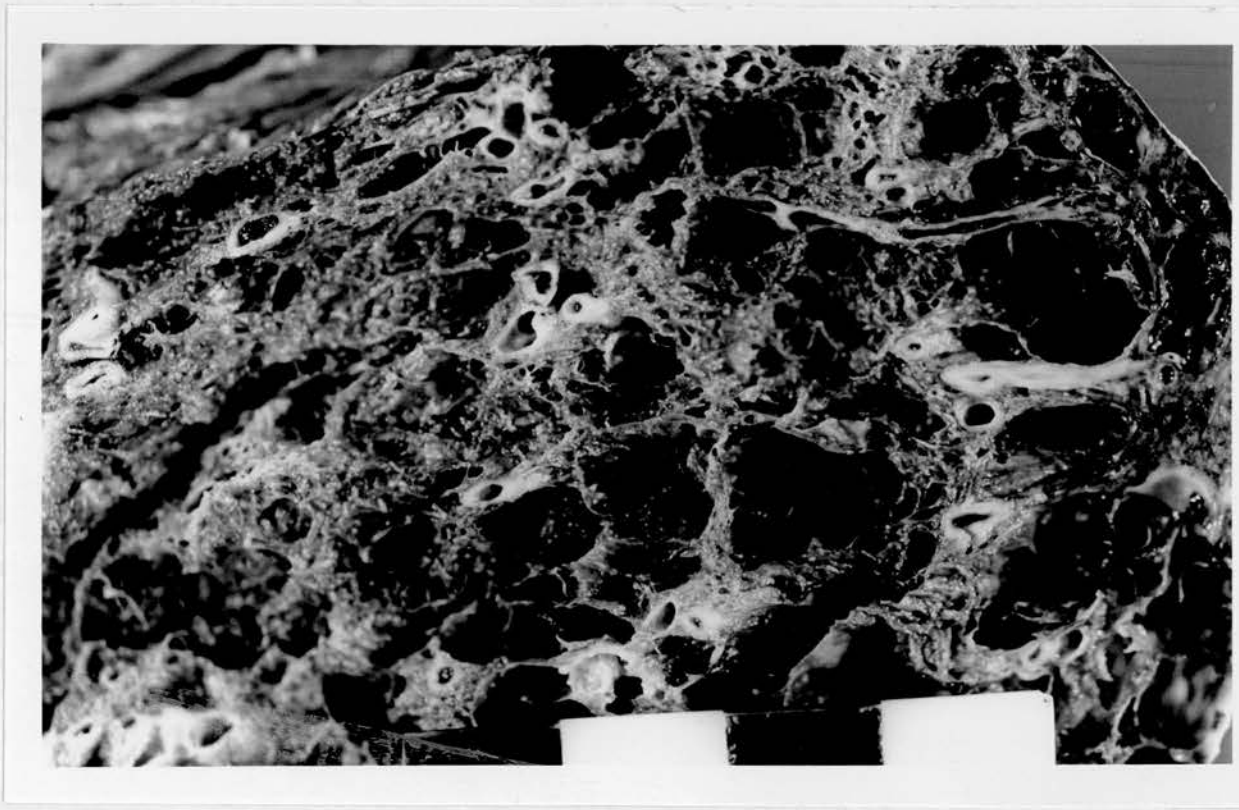
Scale bar: 1cm

Figure 3.3 A lung sample with evidence of mild centriacinar emphysema. The lung destruction can be seen to occur in focal lesions around the respiratory bronchioles, while surrounding lung tissue is normal (compare with the normal lung in Figure 3.1).



Scale bar: 1cm

Figure 3.4 An example of a lung biopsy with severe panacinar emphysema. The greatly enlarged airspaces are fairly uniform. In some regions the amount of destruction has been so great that only blood vessels remain connecting areas of lung tissue such that they appear as strands crossing the spaces.



Scale bar: 1cm

Figure 3.5 A lung sample showing severe centriacinar emphysema. The destruction of lung tissue has been so great that the focal nature of the lesions, which is clearly visible in milder disease, has been lost, making the centriacinar pattern indistinguishable from panacinar emphysema in places.

3.2.1 Clinical information

No significant differences in age, gender, or tumour type were found within the study populations. The median FEV_1 of chronic obstructive airways disease cases was 1.1, and 2.2 for the biopsy study group. Smoking histories were recorded where possible in pack years of exposure, and the median pack years for the chronic obstructive airways disease group was 35, while the biopsy cases had a median of 46 pack years.

3.2.2 Biopsy lung samples

One hundred and fourteen biopsy lung samples contained macroscopic emphysema. Of these samples, 21 contained only panacinar emphysema, 51 contained centriacinar emphysema alone, and 42 samples showed evidence of both centriacinar and panacinar emphysemas. In the lung samples which contained either panacinar or centriacinar emphysema singly, mild disease contributed the majority of cases, and the number of cases with moderate or severe emphysema decreased with increasing severity. In lung biopsies containing both centriacinar and panacinar emphysemas, the number of samples found which contained mild, moderate or severe disease was more evenly distributed. However, cases with both centriacinar and panacinar emphysemas did show a slight trend towards increasing numbers of cases with increasing disease severity.

Microscopic emphysema was found in 40 of the lung samples measured with the fast interval processor.

3.2.3 Autopsy lung samples

Thirty-eight autopsy lung samples were found to contain evidence of macroscopic emphysema. Panacinar emphysema alone was present in 11 autopsy cases, centriacinar emphysema was found to be the single pattern present in 3 autopsy samples, while both centriacinar and panacinar emphysemas were found in 24 lung autopsy cases. The majority of cases with both centriacinar and panacinar emphysemas were classed as severe, unlike the biopsy samples. Insufficient numbers of cases with only one pattern of emphysema were present to identify any trend in severity.

Emphysema cases	Pattern of Disease	Mild	Moderate	Severe	Total
Biopsy cases	Panacinar	12	5	4	21 (18%)
	Centriacinar	34	12	5	51 (45%)
	Both centriacinar and panacinar	10	14	18	42 (37%)
	Total	56 (49%)	31 (27%)	27 (24%)	114
Autopsy cases	Panacinar	1	8	2	11 (29%)
	Centriacinar	0	1	2	3 (8%)
	Both centriacinar and panacinar	4	3	17	24 (63%)
	Total	5 (13%)	12 (32%)	21 (55%)	38
Total cases		61	43	48	152

Table 3.1 Table summarising the pattern and severity of emphysema found in biopsy and autopsy lung samples.

3.2.1 Glutathione S-transferase Pi

GSTP was present in every lung examined. Bronchial epithelial cells were strongly positive with reactivity noted in cytoplasm, brush border and most nuclei (Figure 3.6). Muscle, nerve, serous glands and chondrocytes also expressed GSTP. Terminal bronchioles and both type 1 and type 2 pneumocytes contained GSTP (Figure 3.7), but no significant reactivity was seen in endothelial cells. Alveolar macrophages were variably positive. No differences were noted between smokers and non-smokers.

3.2.2 Glutathione S-transferase Alpha

GSTA was present in the cytoplasm, some nuclei and the brush border of most, but not all, bronchial epithelial cells. Some chrondrocytes stained but alveolar lining cells and macrophages were consistently negative (Figure 3.8).

3.2.3 Glutathione S-transferase Mu

GSTM was much more difficult to detect than the other isoenzyme classes, possibly as a result of lower antibody affinity rather than decreased protein levels. Only two cases of the 21 studied showed unequivocal positive staining but the majority of cases showed weak, indeterminate reactivity. However, the pattern of distribution was the same as for GSTP; that is bronchial epithelium, types 1 and 2 alveolar cells and macrophages (Figures 3.9 and 3.10).

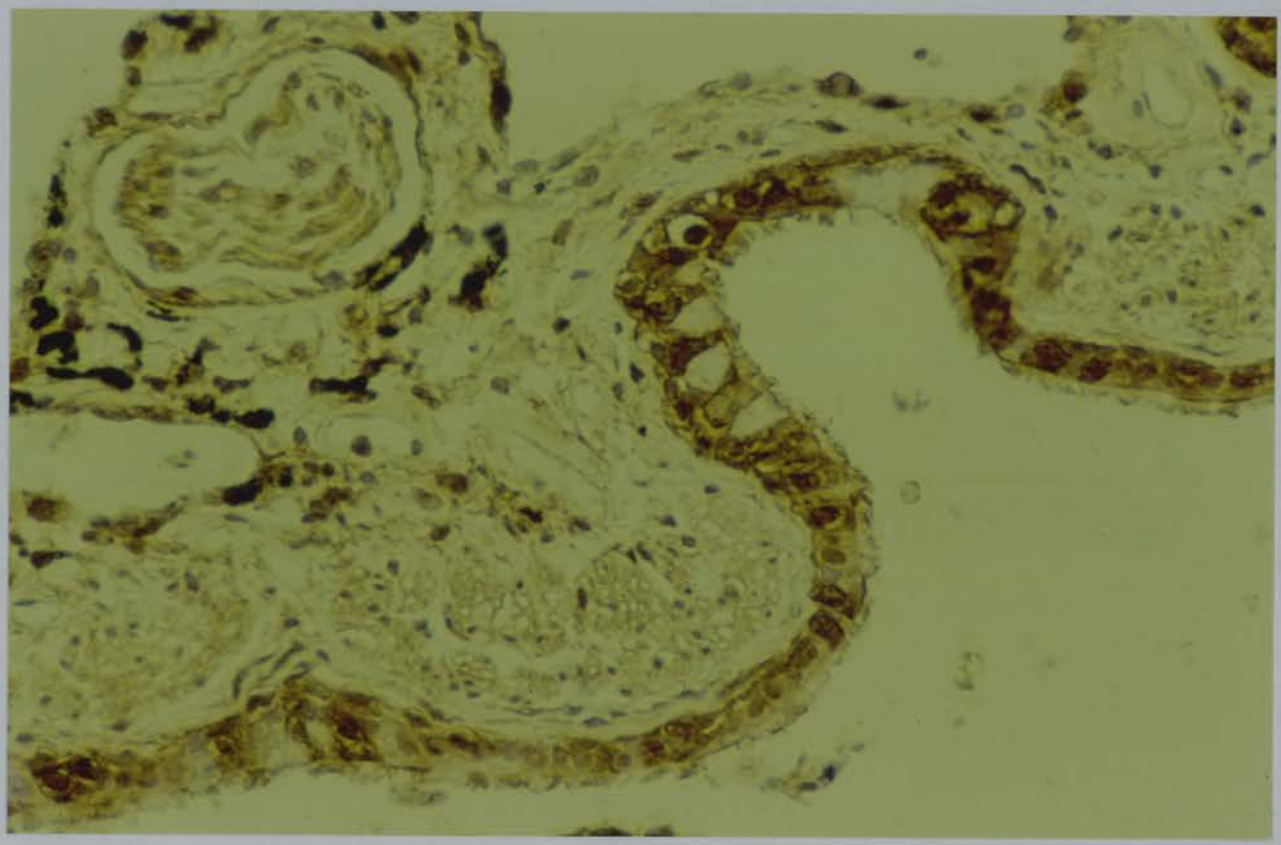


Figure 3.6 Immunohistochemical detection of GSTP in human lung. Staining is localised to bronchial epithelial cells, occasional inflammatory cells and nerves.

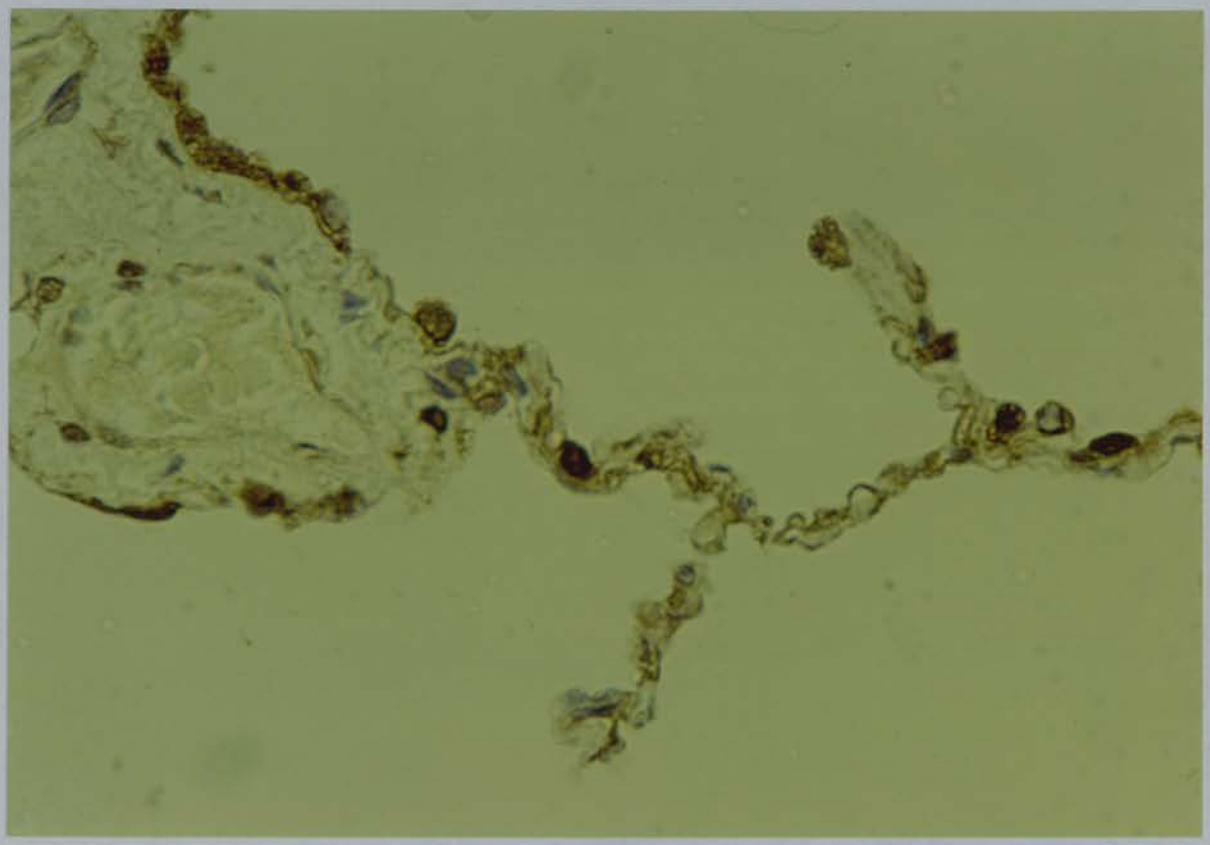


Figure 3.7 Lining cells, particularly type II alveolar cells, strongly expressed GSTP.

Figure 3.8 Immunohistochemical detection of GSTA in human lung. Staining is localised to the brush border of the airway epithelia and is heterogeneous in cells.

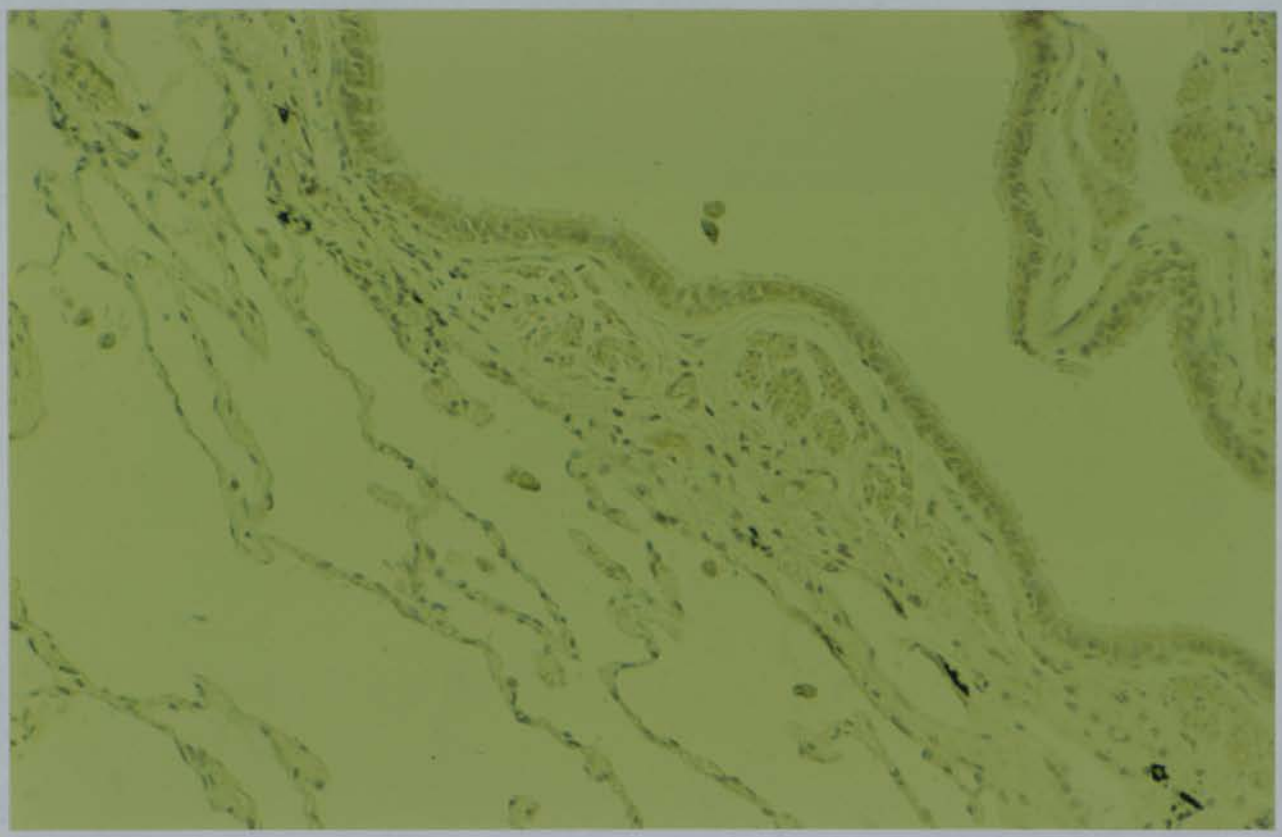


Figure 3.9 Weak immunohistochemical staining localised GSTM to the bronchial epithelium.

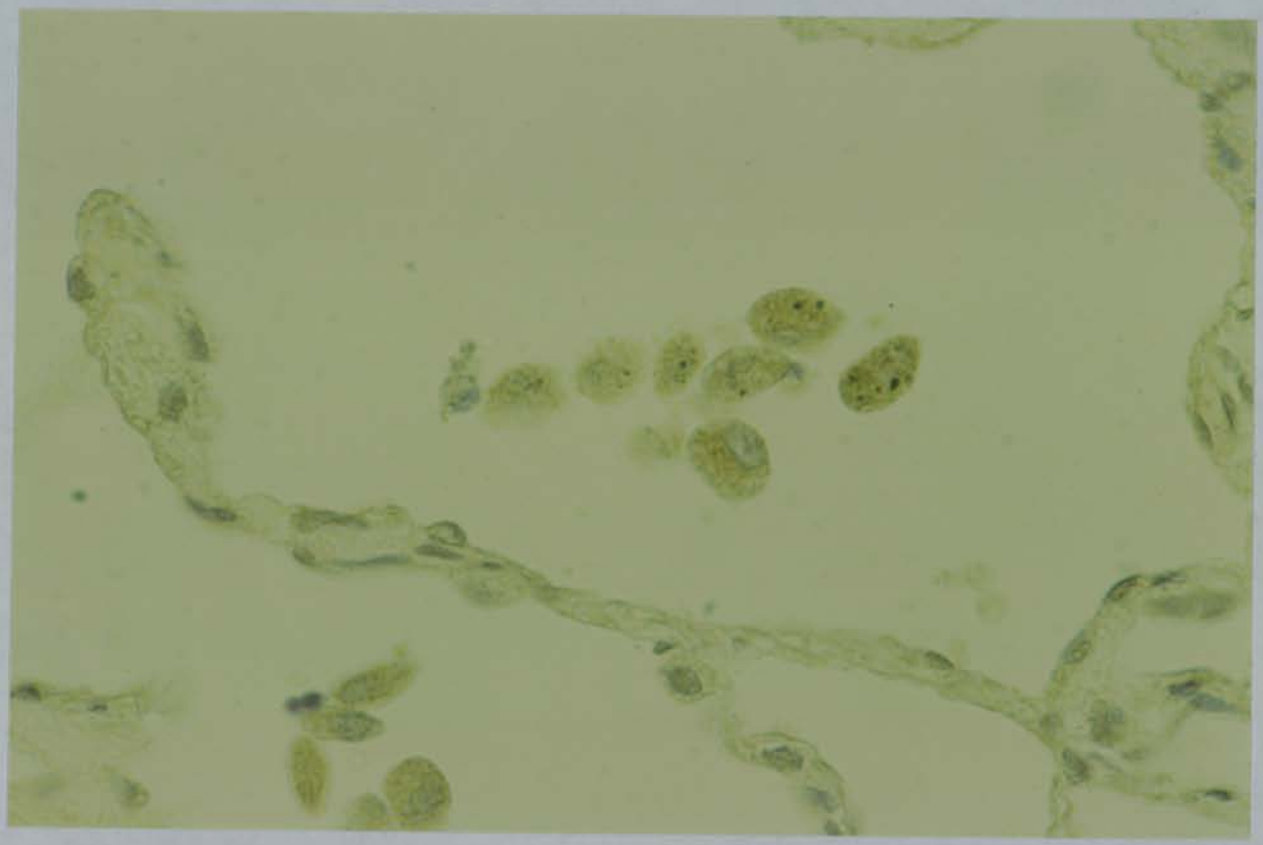


Figure 3.10 Heterogeneous staining of alveolar macrophages for GSTM and weak staining of alveoli.

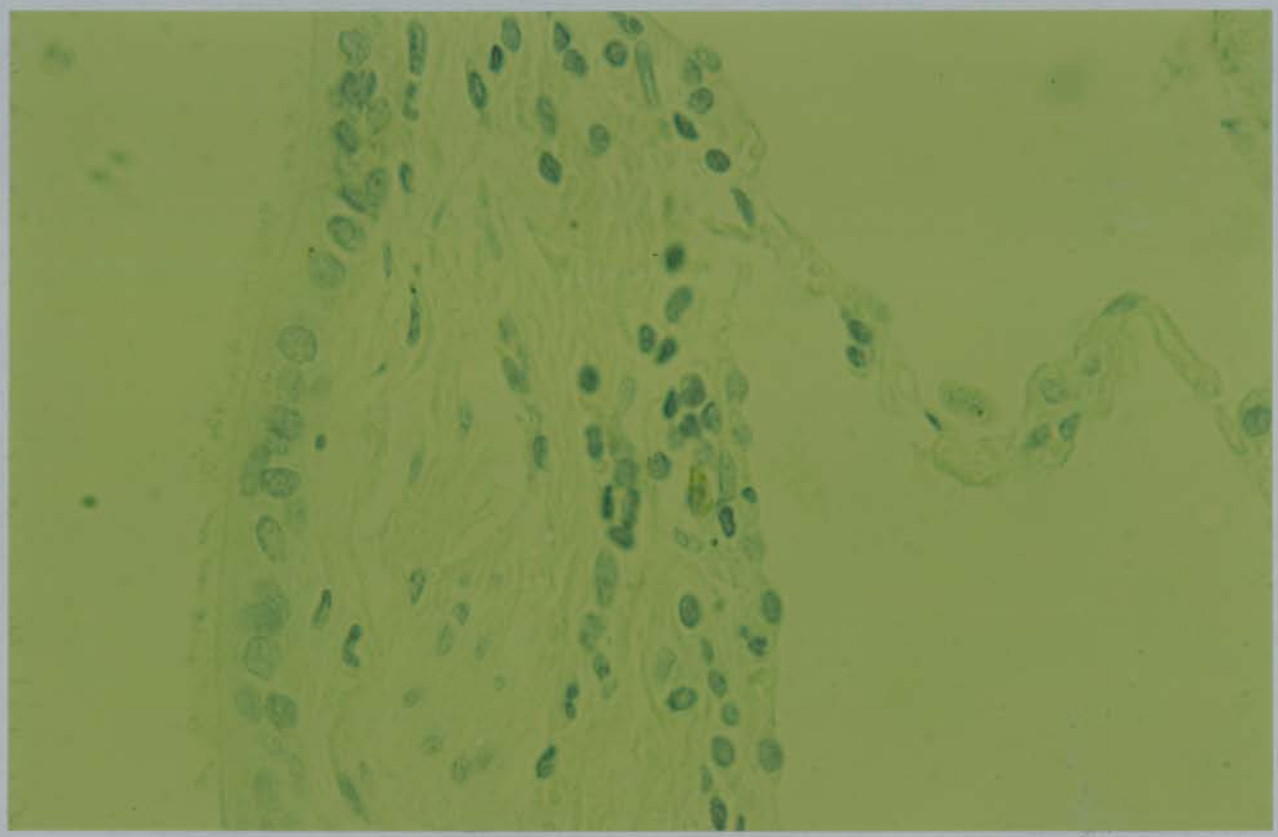


Figure 3.11 Negative control for immunohistochemistry showing no significant reaction product.

3.2.4 Glutathione S-transferase P in bronchoalveolar lavage (BAL) fluid

All four samples showed reactivity with GSTP antibody, although in two cases GSTP appeared to be partly degraded (Figure 3.12). The intensity of the bands varied but the total amount of protein loaded for each case was only approximately equal. The results should therefore not be regarded as quantitative. The BAL fluids used were not noticeably bloodstained macroscopically, and contained few red blood cells by microscopic examination, so it is unlikely that the GSTP present was derived from erythrocytes.

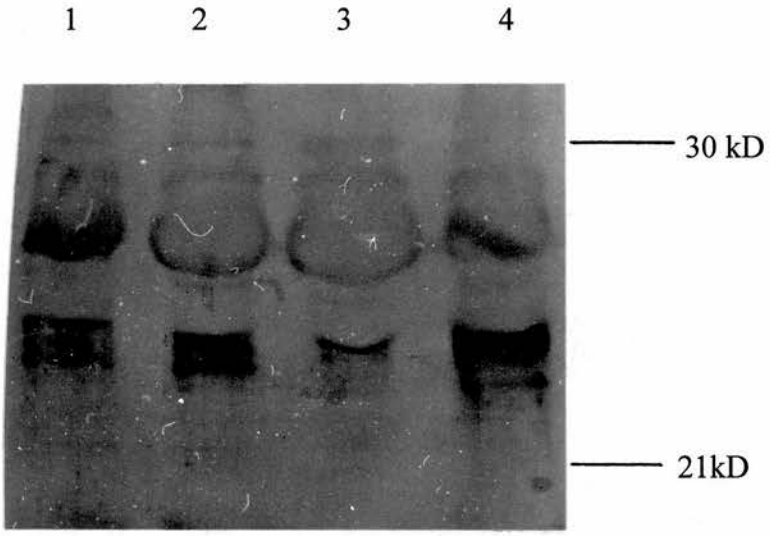


Figure 3.12 Western blot analysis of BAL fluid probed with antibody against GSTP. In lanes 1 and 2 there are lower molecular weight bands reaction with the antibody consistent with degradation of protein.

3.2.5 Comparison of immunohistochemical detection of GSTM with GSTM1 PCR analysis

GSTM1 PCR analysis resulted in specific fragments which were easily interpreted when visualised on agarose gels as described (Figure 3.14). Of 10 DNA samples extracted from archival lung tissue, 7 were null at the GSTM1 locus. There was a poor correlation between GSTM1 genotype and immunophenotype, with 4 cases scoring positive for immunostaining where GSTM1 was absent by PCR (Table 3.2).

Sample	GSTM1 detection by PCR	GST mu detection by immunohistochemistry
1	+	+
2	-	+
3	-	+/-
4	-	-
5	-	+
6	-	+
7	-	+/-
8	+	+
9	-	++
10	+	+/-

Table 3.2 Comparison of GSTM1 genotype with immunohistochemical detection of GSTM.

3.2.6 Detection of human GSTM1 by Western blotting

Western blotting using antibodies to mu class GSTs was carried out on proteins derived from lymphocytes. No expression of GST mu class isoenzymes could be detected using this technique.

3.2.7 Detection of GSTM1 and GSTM4 in human lung tissue by reverse transcription

Both GSTM1 and GSTM4 were detected in 3 lung tissue samples by reverse transcription. GSTM4 was present in all lungs analysed, and GSTM1 expression was demonstrated where genomic PCR analysis indicated that the gene was intact (Figure 3.13).

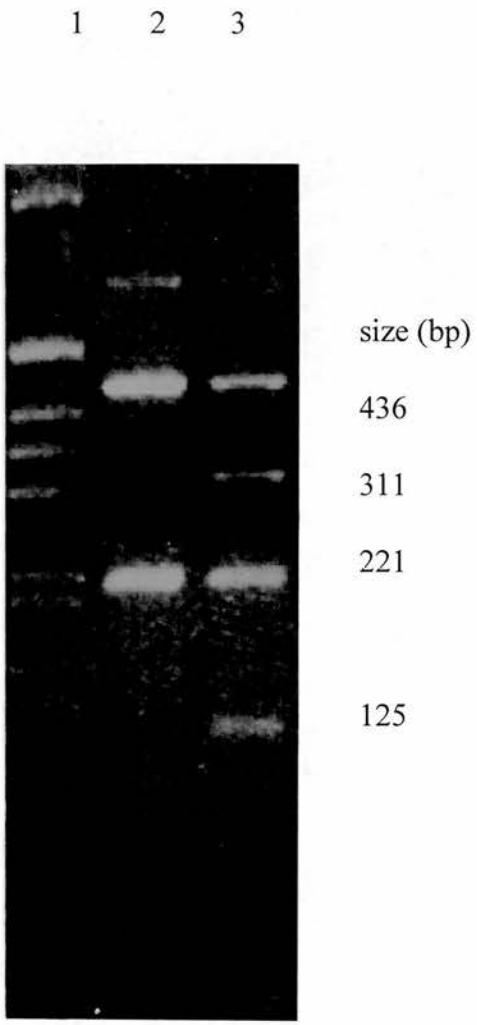


Figure 3.13 Digestion of RTPCR amplimers by *NcoI* restriction enzyme. Lane 1 contains a kilobase ladder. Fragments from a lung sample expressing GSTM4 can be seen in lane 2, while lane 3 shows an *NcoI* digestion of cDNA from a lung sample expressing both GSTM1 and GSTM4.

3.3 Molecular analysis of polymorphic xenobiotic metabolising enzymes in emphysema, chronic obstructive lung disease and lung cancer

3.3.1 Genotyping of GSTM1 lung biopsy cases with emphysema

Following PCR analysis, the reaction products were electrophoresed in agarose gels and fragments of 275 base pairs and 202 base pairs, amplified from GSTM1 and GSTM4 respectively, were visualised. GSTM4 was present in all cases amplified using the assay (Figure 3.14). The results of the GSTM1 genotyping are summarised in Table 3.3.

The control group genotyped for GSTM1 numbered 384 samples, 205 (53%) of which were null for the GSTM1 gene, while 179 (47%) individuals had the intact gene. The GSTM1 genotype of 110 emphysema cases was determined, and 39 (35%) cases were found to have the GSTM1 gene, while 71 (65%) were identified as having the GSTM1 gene deletion. Statistical analysis demonstrated a significant difference between the group of emphysema cases and the control group, with $p=0.038$, an odds ratio of 1.59 and 95% confidence limits from 1.02-2.47.

When the total emphysema cases were sub-divided into groups depending on their pattern of emphysema, 48 cases had centriacinar emphysema alone, panacinar emphysema alone was found in 20 individuals, and both centriacinar and panacinar emphysema were found in 42 samples. The group containing only centriacinar emphysema was found to contain 60% of individuals with deletion of the GSTM1

gene. Of the cases with panacinar emphysema alone, 65% were null for GSTM1. The frequency of GSTM1 deletion in these 2 sub-groups of emphysema did not differ significantly from the control group (p=0.36, p=0.31 respectively). Both centriacinar and panacinar emphysemas were found in 43 samples, and 70% of these individuals were found to have deletion of GSTM1. This proportion of GSTM1 deletion was significantly different to the frequency of the deletion in the control group, p=0.053, with an odds ratio of 1.95, and 95% confidence limits from 0.98-3.86.

3.3.2 GSTM1 genotype and association with lung cancer

A further group of cases comprising 46 lung cancer patients with no evidence of macroscopic emphysema were genotyped for GSTM1. Of these patients, 50% were found to be null for GSTM1, a similar frequency to the 53% of nulled individuals found in the control group.

The total number of cases with lung cancer was subdivided according to lung cancer type and GSTM1 genotypes ascertained for each type of disease. However no significant differences in the frequency of the GSTM1 polymorphism could be demonstrated between the control group and any type of lung cancer (Table 3.4).

3.3.3 GSTM1 genotype and association with COPD

Blood collected from patients attending a chronic obstructive pulmonary disease clinic was genotyped for GSTM1. Of the 80 samples collected, 37 (46%) had the intact GSTM1 gene, while 43 (54%) were null at the GSTM1 locus. This frequency

of genotypes was similar to that of the control population where 53% of individuals lacked the GSTM1 gene.

3.3.4 Genotyping GSTM1 in autopsy lung samples with emphysema

Autopsy samples with evidence of emphysema were collected and genotyped for GSTM1. Of the 38 autopsy lung samples, 24 (63%) had a GSTM1 gene deletion, while 14 (37%) samples had an intact GSTM1 gene. The GSTM1 frequency in these autopsy samples did not differ significantly from the GSTM1 distribution in the control population.

3.3.5 GSTM1 genotype in microscopic emphysema

Forty samples which had been found to have microscopically assessed emphysema were genotyped for GSTM1. Of these samples, 15 (37.5%) had an intact GSTM1 gene, while 25 (62.5%) were null at the GSTM1 locus. The frequency of deletion of GSTM1 in these cases were not significantly different from the controls.

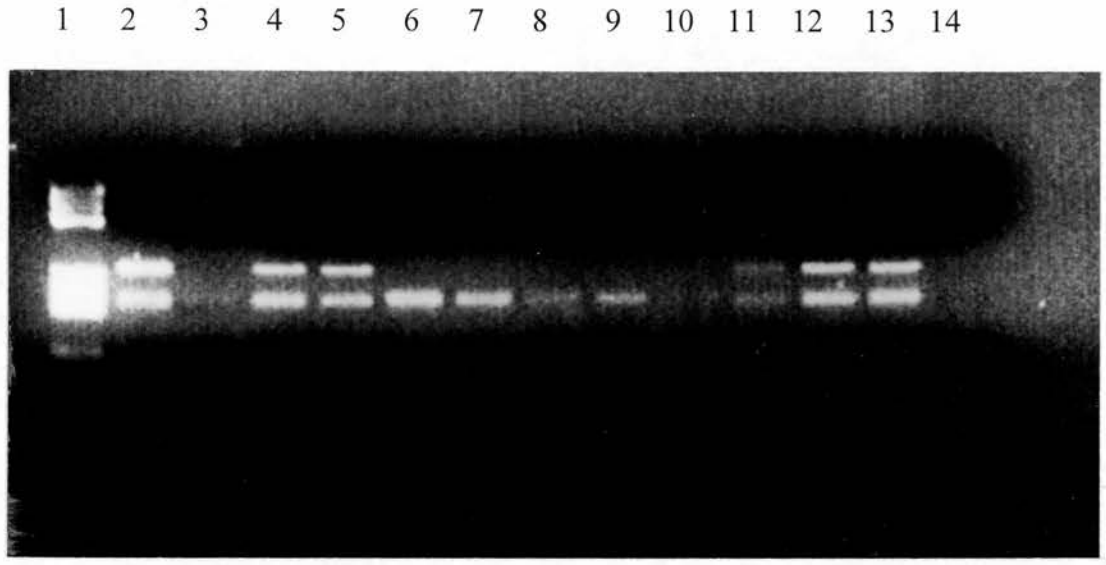


Figure 3.14 GSTM1 and GSTM4 PCR products amplified from control blood samples. The 202bp fragment amplified from GSTM4 is present in every sample, and the 275bp GSTM1 fragment is present in only some cases (lanes 2, 4, 5, 11, 12, and 13). A negative control reaction containing water and no template was loaded in lane 14), and lane 1 contains DNA molecular weight marker V (Boehringer Mannheim, UK).

	GSTM1 present	GSTM1 deleted	Total cases	χ^2 versus control	p value	odds ratio	95% confidence limits
Emphysema Cases	39 (35%)	71 (65%)	110	4.32	0.038	1.59	1.02-2.47
Centriacinar only	19 (40%)	29 (60%)	48	0.85	0.357	1.33	0.72-2.45
Panacinar only	7 (35%)	13 (65%)	20	1.03	0.310	1.62	0.63-4.15
Both centriacinar and panacinar emphysemas	13 (31%)	29 (69%)	42	3.75	0.053	1.95	0.98-3.86
Lung cancer without emphysema	23 (50%)	23 (50%)	46	0.19	0.664	0.87	0.47-1.61
COPD cases	37 (46%)	43 (54%)	80	0.004	0.953	1.01	0.63-1.64
Autopsy emphysema cases	14 (37%)	24 (63%)	38	1.33	0.249	1.50	0.75-2.99
Microscopic emphysema	15 (37.5%)	25 (62.5%)	40	1.33	0.249	1.50	0.75-2.98
Control	179 (47%)	205 (53%)	384	-	-	-	-

Table 3.3 Frequency of GSTM1 deletion polymorphism in lung cancer cases when grouped according to the presence of emphysema.

Lung Cancer	GSTM1 present	GSTM1 deletion	Total	χ^2 versus control	p value	odds ratio	95% confidence limits
Squamous Carcinoma	22 (40%)	33 (60%)	55	0.85	0.357	1.31	0.74-2.33
Adenocarcinoma	18 (41%)	25 (58%)	43	0.35	0.553	1.21	0.64-2.30
Adenosquamous Carcinoma	0	4 (100%)	4	FT*	0.128	7.86	0.42-147.12
Small Cell Undifferentiated	6 (40%)	9 (60%)	15	FT*	0.793	1.31	0.46-3.75
Large Cell Undifferentiated	3 (43%)	4 (57%)	7	FT*	1.00	1.16	0.26-5.27
Other	11 (55%)	9 (45%)	20	0.54	0.464	0.71	0.29-1.75
Total	60 (42%)	84 (58%)	144	1.03	0.309	1.22	0.83-1.80
Control Population	179 (47%)	205 (53%)	384	-	-		

*Statistics test used was Fishers exact test.

Table 3.4 Table showing the frequency of GSTM1 genotypes in lung cancer cases

3.3.6 CYP1A1 genotyping of emphysema samples

Of the 129 lung cancer samples studied, 35 were assessed to have no macroscopic emphysema, 34 showed centriacinar patterns of damage, 17 samples had panacinar emphysema, and 35 lung biopsies had both centriacinar and panacinar forms of emphysema

PCR analysis of control blood samples yielded specific fragments which were easily visualised on agarose gels (Figure 3.15). DNA extracted from paraffin embedded lung tissue generally amplified successfully, however there was an increased tendancy towards smearing and smaller fragments (Figure 3.16). Each CYP1A1 amplimer analysed had a diagnostic *NcoI* restriction enzyme site which enabled distinction of the CYP1A1 genotype of the individual, and a second constant control *NcoI* site which served to check the efficacy of the enzyme (Figure 3.17).

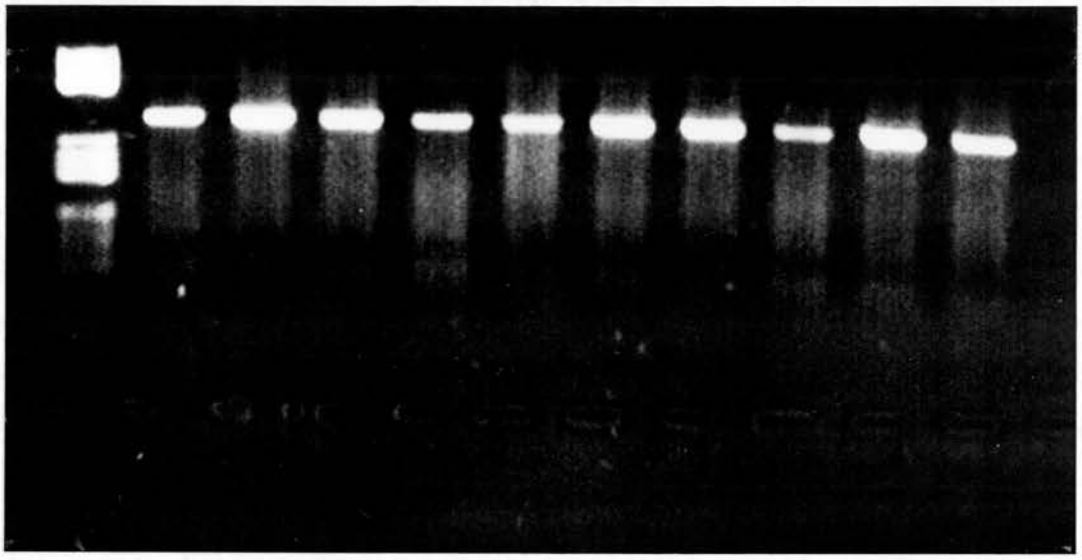


Figure 3.15 CYP1A1 PCR products amplified from control DNA samples (extracted from blood). A product of 322bp is amplified in each case. DNA molecular weight marker V (Boehringer Mannheim, UK) is loaded in the left hand lane.

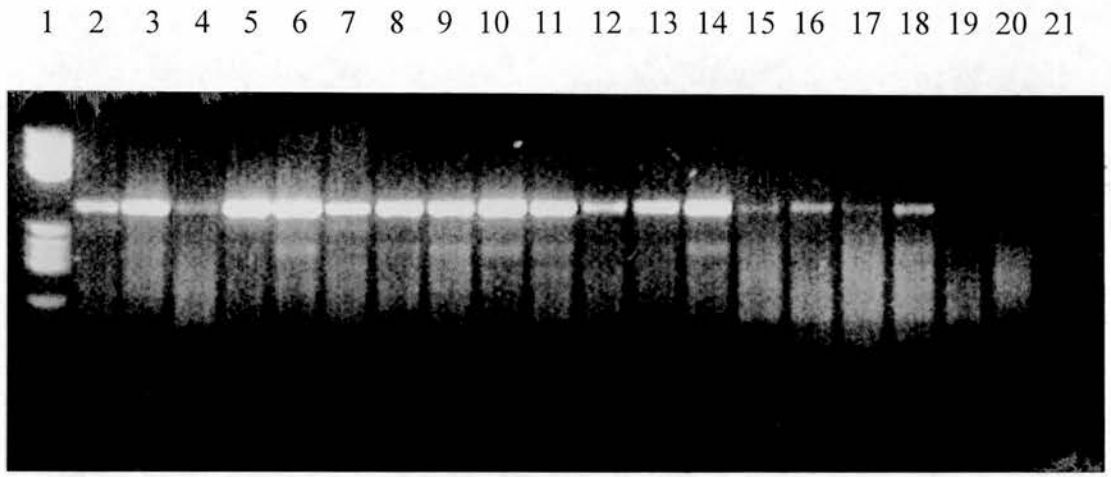


Figure 3.16 CYP1A1 PCR products amplified from DNA extracted from paraffin embedded lung tissues. The expected 322bp CYP1A1 fragment is present in lanes 2-17, but with DNA extracted from these samples there was an increased chance of amplifying smaller fragments and smearing. Lane 1 contains DNA molecular weight marker V (Boehringer Mannheim, UK). A negative control reaction containing water and no template has been loaded in lane 21 to act as a negative control.

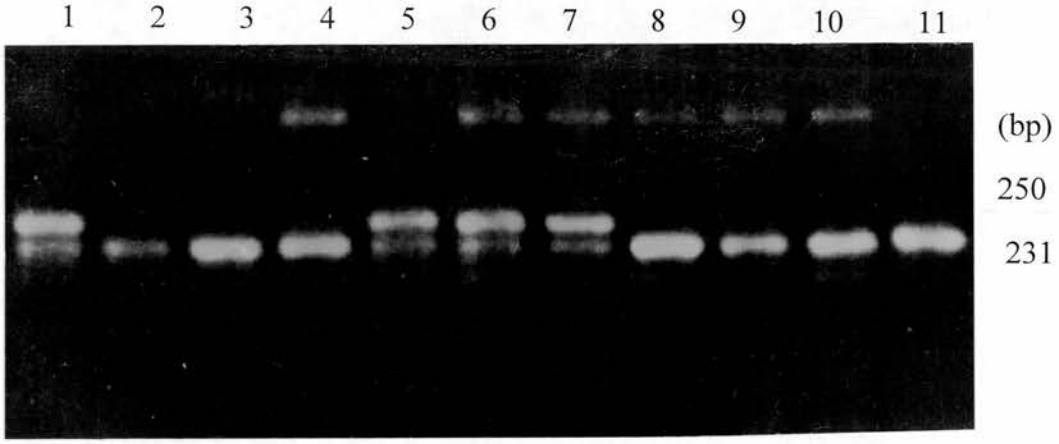


Figure 3.17 Results of a *NcoI* digestion of the CYP1A1 amplimer. The 2 bands represent the 2 alleles of the gene, the higher molecular weight fragment (250bp) representing the uncut valine allele of the gene, and the lower molecular weight band (231bp) representing the cut isoleucine allele of CYP1A1. Lanes 1,5,6 and 7 therefore represent heterozygotes for the 2 alleles, while lanes 2,3,4,8,9,10 and 11 contain digestions from individuals homozygous for the common isoleucine allele. A non-specific higher molecular weight band can be seen in lanes 4,6,7,8,9 and 10.

The control group numbered 281 samples, of which 87% were homozygous for the Ile^{462} allele, 12% were heterozygous, and 1% were homozygous for the valine allele of the CYP1A1 gene (Table 3.5). In comparison, the 121 lung resection cases demonstrated 82% homozygosity at the Ile^{462} allele, heterozygosity in 16% of individuals, and 2% individuals with Val^{462} homozygosity. This group was not significantly different from the control population (Table 3.4).

The CYP1A1 genotype was identified in 35 lung samples which showed evidence of microscopic emphysema. Of these samples, 24 (68%) were homozygous for the Ile^{462} allele, 9 (26%) were heterozygotes, and 2 (6%) were Val^{462} homozygotes. This distribution of the CYP1A1 polymorphism is significantly different from the control population frequencies (χ^2=11.15, p=0.001), with an odds ratio of 3.06 and 95% confidence intervals of 1.54-6.07.

In the clinical group of chronic obstructive pulmonary disease patients, of the 80 samples, 80 % were homozygous for Ile^{462}, and 20% were heterozygous for the polymorphism. No individuals homozygous for the valine allele were found in this disease group, and no significant difference between this study group and the controls was seen (Table 3.5).

In order to investigate the CYP1A1 gene polymorphism and susceptibility to emphysema, the total lung cancer study group was divided according to the pattern of emphysematous damage in the lung (Table 3.6). When this was done, 35 samples

were found to show no evidence of emphysema, and of these, 91% were Ile^{462} homozygotes and 9% were heterozygotes. Centriacinar emphysema only was found in 34 cases, and of these, 73.5% were homozygous for the valine allele and 26.5% were Ile/Val. Panacinar emphysema alone was present in 17 samples, 88% of which were Ile^{462} homozygotes, 6% were Ile/Val, and 6% were homozygous for the Val^{462} allele. Both panacinar and centriacinar emphysemas were found in 35 of the lung cancer samples, and homozygotes for the isoleucine allele accounted for 77% of these cases, 20% were heterozygotes, and 3% were Val^{462} homozygotes. No significant differences were found between the control population and the patient groups with either centriacinar or panacinar emphysema alone and the patients with both centriacinar and panacinar emphysemas.

3.3.7 Genotyping of CYP1A1 in lung cancer cases

The lung cancer cases were subdivided according to type of lung cancer, and the frequencies obtained for each type of cancer are shown in Table 3.7. No significant differences were demonstrated to exist between the frequency of the CYP1A1 polymorphism in the control population and the frequencies obtained for the groups of patients with different types of lung cancer.

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	Ile/Ile geno-type	Ile/Val geno-type	Val/Val geno-type	Total cases	$\chi 2$ versus control*	p value	Odds ratio	95% confidence limits
Control	245 (87%)	33 (12%)	3 (1%)	281	--	--	--	--
All lung cancers	99 (82%)	20 (16%)	2 (2%)	121	2.08	0.15	1.48	0.87-2.52
Cancer with no emphysema	32 (91%)	3 (9%)	0	35	0.71	0.40	0.60	0.18-1.99
Cancer with emphysema	67 (78%)	17 (20%)	2 (2%)	86	4.87	0.03	1.86	1.06-3.26
Microscopic emphysema	24 (68%)	9 (26%)	2 (6%)	35	11.15	0.001	3.06	1.54-6.07
Chronic Obstructive Pulmonary Disease	64 (80%)	16 (20%)	0	80	1.66	0.20	1.49	0.81-2.72

Table 3.5 Table showing CYP1A1 genotypes for the disease groups and control group for statistical comparison. *Comparison of allele frequencies.

	Ile/Ile genotype	Ile/Val genotype	Val/Val genotype	Total cases	$\chi 2$ versus control*	p value	Odds ratio	95% confidence limits
Centriacinar alone	25 (73.5%)	9 (23.5%)	0	34	3.42	0.065	2.04	0.94-4.42
Panacinar alone	15 (88%)	1 (6%)	1 (6%)	17	FT**	0.725	1.30	0.38-4.44
Both centri- and panacinar	27 (77%)	7 (20%)	1 (3%)	35	3.11	0.08	1.98	0.91-4.28

Table 3.6 Table showing CYP1A1 genotypes of cases with emphysema and lung cancer divided by pattern of emphysema. *Comparison of allele frequencies. **Stastical test used was Fishers exact test.

Lung cancer	Ile/Ile geno-type	Ile/Val geno-type	Val/Val geno-type	Total cases	$\chi 2$ versus control*	p value	Odds ratio	95% confi-dence limits
Squamous carcinoma	35 (79.5%)	7 (16%)	2 (4.5%)	44	3.31	0.07	1.92	0.94-3.91
Adenocar-cinoma	27 (84%)	5 (16%)	0	32	0.07	0.80	1.14	0.43-3.00
Adeno-squamous Carcinoma	3 (100%)	0	0	3	FT**	1.00	1.02	0.06-18.44
Small Cell Carcinoma	5 (62.5%)	3 (37.5%)	0	8	FT**	0.10	3.09	0.85-11.32
Large Cell Carcinoma	2 (67%)	1 (33%)	0	3	FT**	0.36	2.68	0.31-23.54
Other	18 (95%)	1 (5%)	0	19	FT**	0.50	0.36	0.05-2.69
Total	90 (82%)	17 (16%)	2 (2%)	109	1.60	0.21	1.43	0.82-2.49

Table 3.7 Table showing distribution of CYP1A1 genotypes among lung cancer cases. *Comparison of allele frequencies. **Statistical test used was Fishers exact test.

3.3.8 Sequence analysis of CYP1A1 gene for polymorphism confirmation

Sequence analysis of the CYP1A1 PCR product confirmed that the PCR assay and subsequent restriction enzyme analysis identified individuals with the A to G substitution polymorphism of CYP1A1 (Figure 3.18).

3.3.9 Detection of novel Thr-Asn mutation in CYP1A1 gene

Following sequence analysis of the CYP1A1 PCR product of one individual, it was discovered that the expected A to G substitution, which results in the replacement of a valine for an isoleucine amino acid at position 462, was not present, despite this individual being genotyped as having the mutation by restriction enzyme analysis. The sequencing data derived from this individual identified a novel CYP1A1 mutation, a C to A substitution, 2 base pairs upstream of the known valine polymorphism. This mutation would result in the substitution of an asparagine for a threonine amino acid residue.

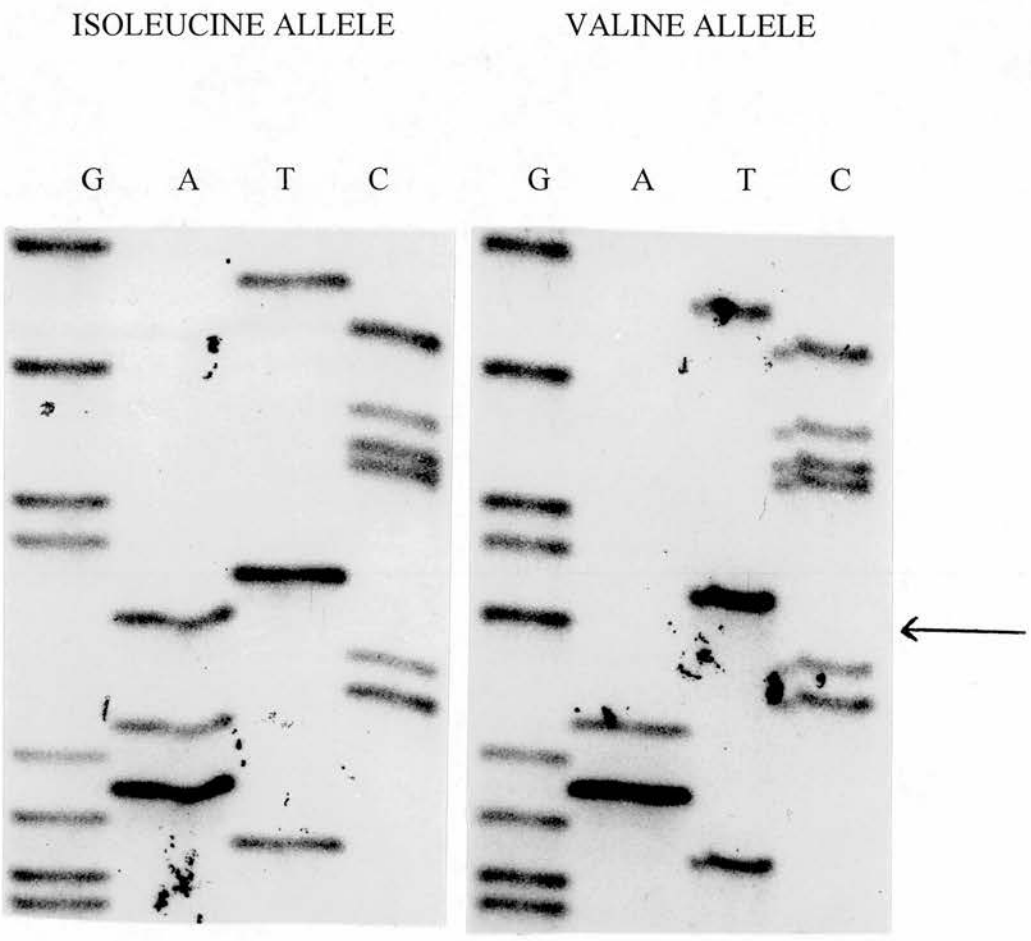

Figure 3.18 Sequence analysis of the CYP1A1 isoleucine to valine polymorphism. The A to G substitution can be clearly seen when the polymorphic valine allele is sequenced and compared to the sequence of the more common isoleucine allele.

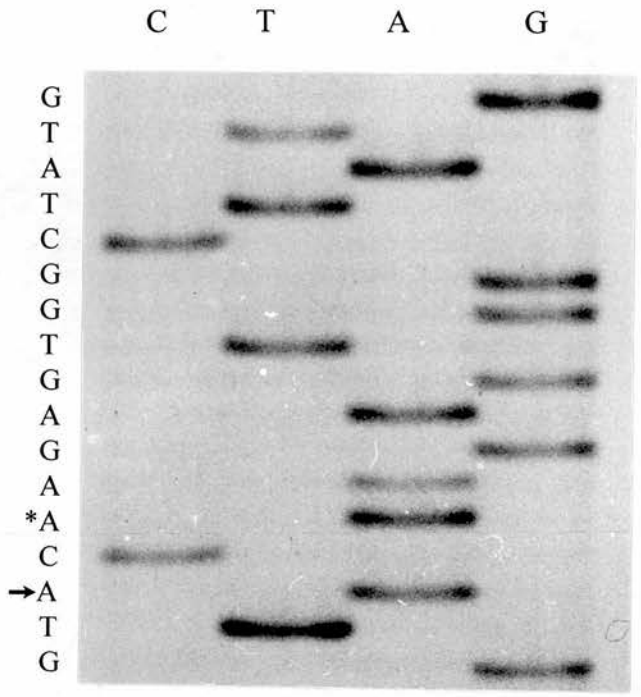

Figure 3.19 Detection of a novel variant of the CYP1A1 gene by cloning and sequence analysis. A clear C to A substitution (*A) was identified in one sample which had been genotyped as homozygous for the polymorphic valine allele using PCR and *NcoI* restriction enzyme analysis. The presence of the more common A (→A) as opposed to the polymorphic G base pair is clear, indicating that the false genotyping obtained for the isoleucine to valine polymorphism resulted from disruption of the *NcoI* site by this novel CYP1A1 sequence variation.

3.3.10 PCR analysis of control population for Thr-Asn polymorphism

Any identification of Ile^{462} using the previous CYP1A1 PCR and *NcoI* restriction enzyme assay must be correct, since the enzyme can cut this gene sequence, but either the Val^{462} or the Asn^{461} variants will destroy this restriction enzyme site. Where destruction of the restriction enzyme site was demonstrated in the previous assay this was subscribed to the polymorphic valine allele, however this destruction may be the result of the Thr-Asn^{461} change, which may be a common variant of the CYP1A1 gene.

Genotyping was carried out on 26 control samples which had previously been identified as heterozygotes for the Ile-Val^{462} polymorphism. The individual who was demonstrated to have the novel CYP1A1 variant through sequencing had previously been genotyped as a homozygote for the valine allele. This individual was therefore also genotyped with the new assay to determine whether this person was homozygous for the novel mutation, or was a heterozygote with an allele containing the valine variant of the gene.

With this analsysis, 14 individuals were heterozygous for restriction by *Sau96I* enzyme. Since previously these individuals had been demonstrated to have the

isoleucine variant, their genotype must be isoleucine/asparagine. The CYP1A1 fragments amplified from 12 individuals were found to digest completely with *Sau96I* indicating that their original genotyping as heterozygotes for the isoleucine and valine alleles was correct. The individual who had previously been classified as a valine homozygote was found to have two copies of the Asn^{461} allele of the gene.

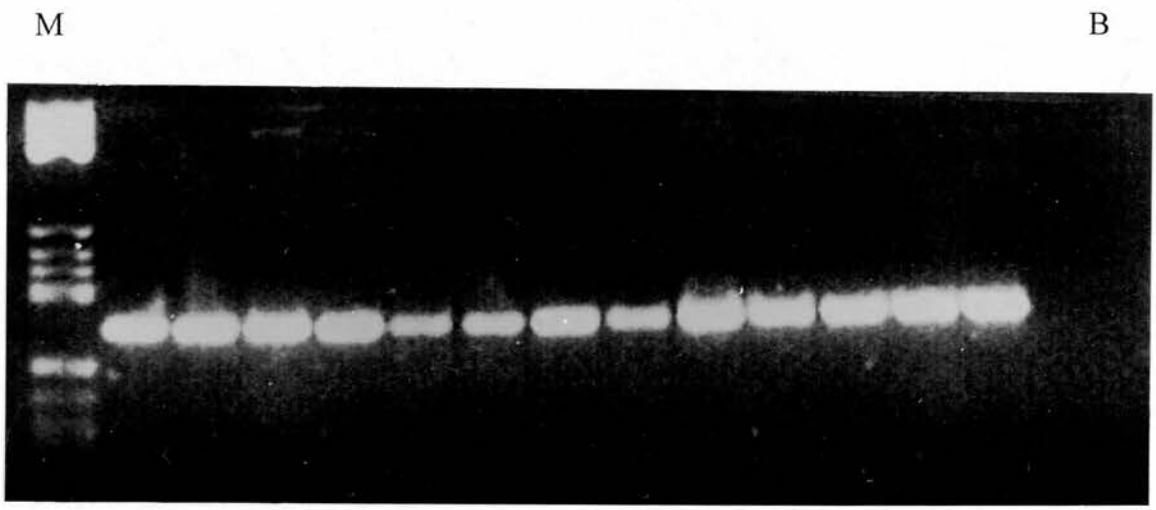

Figure 3.20 Photograph of the amplimers obtained using PCR for the novel Thr-Asn polymorphism of CYP1A1. The fragment size is 153bp, M refers to DNA molecular weight Marker V, B refers to the negative control for the PCR (no DNA added).

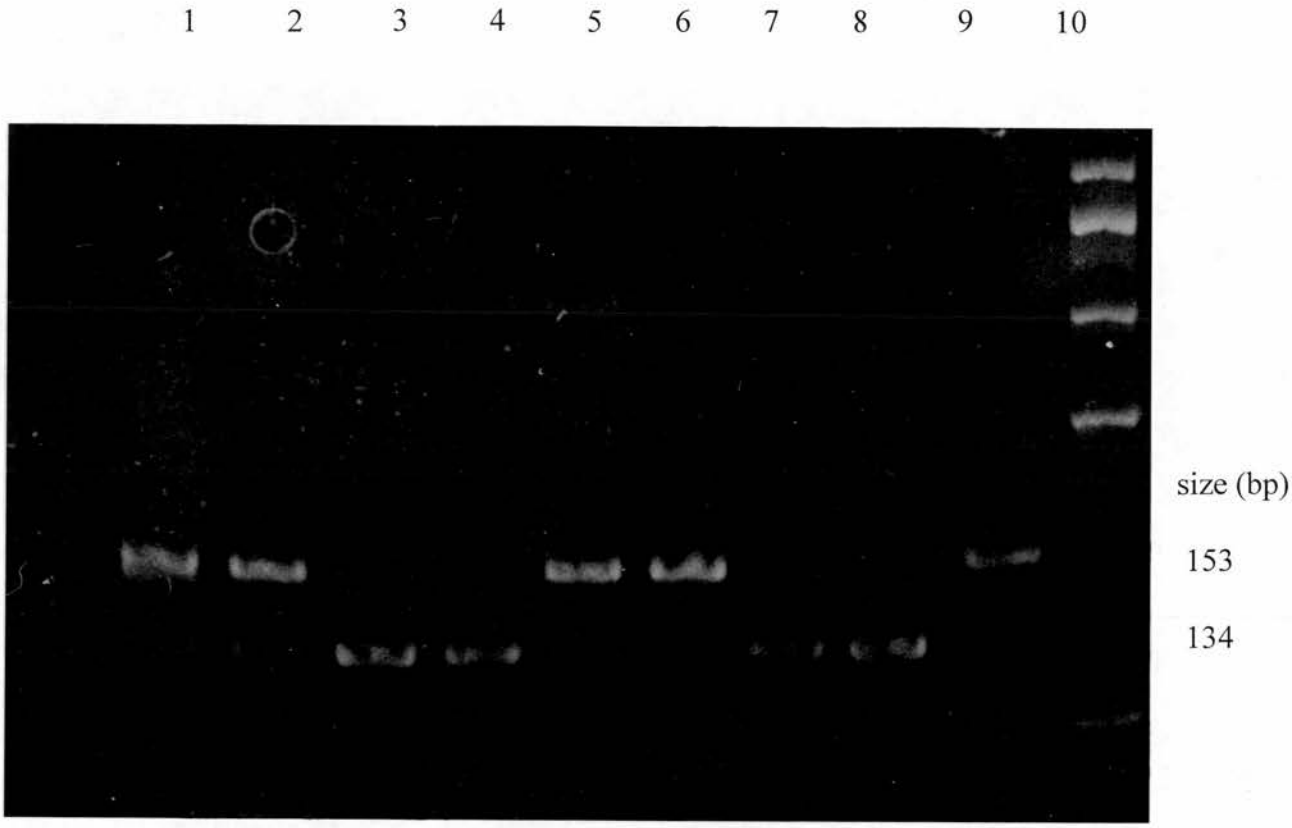

Figure 3.21 Sau96I digestion of the PCR products for the novel Thr-Asn polymorphism. Lanes 1,2,5 and 9 show individuals heterozygous for the novel Asn polymorphism, lanes 3,4,7 and 8 show individuals who do not have the Asn allele of CYP1A1, while the homozygote for the Asn polymorphism is in lane 6. Lane 10 contains DNA molecular weight marker ϕX174.

DISCUSSION

4.1 Distribution of emphysema in biopsy and autopsy cases

As can be seen in Table 3.1, panacinar and centriacinar emphysemas were not evenly distributed between the autopsy and biopsy cases. In biopsy cases, more centriacinar emphysema and both centriacinar and panacinar emphysemas together were present, with less cases containing panacinar emphysema alone. Moreover, a trend towards milder disease was also identified, with moderate and severe disease being less common than mild disease in the biopsy cases.

The autopsy cases showed a different distribution in disease pattern and severity to that seen in biopsy cases. Most autopsy samples contained both centriacinar and panacinar emphysemas, while panacinar emphysema was more common than centriacinar emphysema where a single form of the disease was present in the lung. Indeed, few autopsy cases were found to contain only centriacinar emphysema. In autopsy cases there seemed to be a tendancy towards increased severity, unlike the trend identified in biopsy cases. However, since lower numbers of autopsy cases were assessed, this trend is perhaps less credible than that identified in the biopsy cases.

The uneven distribution both of emphysema pattern and severity in autopsy and biopsy cases is not surprising. Since patients undergoing surgery for biopsy need to

be of a certain level of fitness to survive the operation, these samples would be expected to contain generally milder disease than might be expected in autopsy samples. Autopsy samples would generally be expected to contain disease of a more severe nature since in many of these cases emphysema may have been the cause of death either directly, or as a contributory factor. Furthermore, while many autopsy cases may contain emphysema, selection of samples for a study of emphysema would be more likely to have occurred for interesting cases with more severe emphysema.

Kim and colleagues (1991) demonstrate that the loss of elastic recoil associated with chronic obstructive pulmonary disease correlates with increased airspace size in the parenchyma rather than increased airspaces in centrilobular lesions. It might therefore be hypothesized that panacinar emphysema is more debilitating to lung function than centriacinar emphysema. With this hypothesis in mind, autopsy cases might be expected to contain more panacinar emphysema than biopsy samples which have been obtained from individuals who do not suffer from severe chronic obstructive pulmonary disease. Individuals with centriacinar emphysema will be less likely to suffer decreased lung function as a result of their disease and will therefore be judged capable of surviving biopsy surgery, explaining the increased numbers of individuals with this type of emphysema in the biopsy group.

If emphysema has contributed significantly to the cause of death, there are several reasons why it might be expected that the lungs would contain both panacinar and centriacinar emphysema. Firstly, in more severe disease both forms of emphysema

are likely to be present, since increased damage reflects increased exposure (and/or susceptibility) to cigarette smoke. Secondly, both panacinar and centriacinar emphysema contribute towards decreased lung function, therefore the number of samples with both types of disease would be expected to be higher in autopsy cases than in biopsy cases. Indeed, when the proportion of cases with both types of emphysema in the biopsy and autopsy groups is compared, an increased percentage of these cases is seen in autopsy samples.

4.2 Immunohistochemical detection of GSTs in human lung tissue

The immunohistochemical findings of previous work carried out in the laboratory suggested that GST-mediated protection against xenobiotic and oxidative stress in the lung can be divided into two functional compartments (Cantlay *et al*, 1994). In proximal airways all three cytosolic forms GSTP, A and M were present in lining epithelium, whereas the distal alveolar compartment contained GSTP and GSTM but no GSTA in both type 1 and 2 pneumocytes as well as macrophages. This is consistent with biochemical studies which showed that GSTP is the predominant lung isoenzyme (Ketterer *et al*, 1992; Carmichael *et al*, 1993). In a previous study of GST in human lung development (Cossar *et al*, 1990), all isoenzymes were identified, with GSTP the predominant isoenzyme. The levels of GSTP were higher during fetal development, with strong reactivity in bronchi and protoalveoli, and fell during late gestation. In another study of only 3 human lungs (Awasthi *et al*, 1987), GSTP, GSTA and GSTM were all reportedly present in alveoli. This is inconsistent with the work of Fryer and colleagues in fetal lungs (1986) and the results obtained

in our laboratory. The discrepancy may have resulted because an immunofluorescence assay was used in the study of Awasthi, which is more sensitive but also produces a higher background.

In previous studies of localisation of GST in mouse and rat lungs (McLellan *et al*, 1992; Coursin *et al*, 1992), GST was restricted to bronchial epithelial cells, including the brush border, with virtually no GST identifiable in alveoli. In addition, GSTP in rat lung was also identified as an extracellular protein in association with elastin (Coursin *et al*, 1992). This was apparent in electron but not light microscopic preparations. The results obtained in our laboratory provided no immunohistochemical evidence for extracellular interstitial GST but the presence of GSTP was demonstrated in BAL fluid. In a previous study both GSTA and GSTP were found in BAL fluid of tumour bearing lungs (Howie *et al*, 1990b).

The presence of GSTP in alveolar macrophages and BAL is interesting. GSTP functions as a homodimer and it can be readily inactivated by oxidation of reduced sulphydryl groups (Mulder & Tekoppele, 1988). It may therefore function as a "sacrificial reactive protein" in addition to being a GST dependent enzyme. Indirect support for this suggestion is provided from a study where cigarette smoke reduced GSTP activity in alveolar macrophages (Petruzelli *et al*, 1988), despite the fact that cigarette smoke contains many chemicals which might increase GSTP expression. In these cells GSTP activity may have been reduced by oxidative degradation rather than by reduced expression of the protein.

Cigarette smoke contains a plethora of chemicals, many of which act as substrates for GSTP, GSTA and GSTM. Proximal airways contained GSTA which is involved in selenium-independent glutathione peroxidation reactions, and which may detoxify DNA and lipid hydroperoxides (Ketterer *et al*, 1988). Its presence in the brush border is therefore suggestive of a role for GSTA in the protection of bronchial epithelium. Most acute damage following cigarette smoke inhalation is seen in small distal airways and alveoli (Harley, 1988), a site lacking this selenium-independent peroxidase protection. In individuals null at the GSTM1 locus it therefore follows that the level of protection in distal airways afforded by GSTs is significantly reduced. Several reports have suggested that the expression of GSTM in lung shows evidence of polymorphism which is consistent with the gene expressed being GSTM1 rather than GSTM4 (Fryer *et al*, 1986; Ketterer *et al*, 1992). However, in one study of 10 lungs, no evidence of polymorphic expression was seen although the level of GSTM catalytic activity was markedly variable between cases (Carmichael *et al*, 1993). The immunohistochemical expression carried out in our laboratory could not differentiate specifically the polymorphic enzyme GSTM1 from the non-polymorphic GSTM4.

Previous studies of polymorphic xenobiotic metabolising enzymes had identified a role for GSTM1 deletion in influencing susceptibility to lung cancer, and it was therefore hypothesised that this enzyme deficiency might also influence

susceptibility to emphysema, another lung disease which has a strong association with cigarette smoking. If the GSTM1 polymorphism is involved in susceptibility to lung disease, the expression of this enzyme is of primary interest. GSTM1 is highly expressed in the liver, therefore a deficiency in metabolism might influence disease through systemic circulation. However, if GSTM1 expression occurs in lung tissue, the deficiency in metabolism might be expected to have a direct effect. Any difference in expression may influence disease susceptibility with regard to the type of disease and the region of the lung involved. For example in panacinar emphysema, systemic factors influencing disease may be of particular importance, since deficiency of α1-antiprotease, the major antiprotease which is present in serum predisposes individuals to develop emphysema of the panacinar type (Guenter *et al*, 1968).

In order to determine whether expression of GSTM1 in human lung samples could be shown by immunohistochemistry, the GSTM1 genotype of lung samples was compared with immunohistochemistry of the GSTM class enzymes. The GSTM1 genotype was determined by PCR in 10 archival lung samples which had been included in the previous immunohistochemical analysis. The GSTM1 genotype showed poor correlation with staining for GSTM class enzymes. This may be explained by the specificity of the PCR assay for GSTM1 only, while the immunohistochemistry may identify any of the mu class enzymes, including GSTM4, which is expressed in lung (Antilla *et al*, 1993). Since

immunohistochemical staining of lung tissue for GSTM enzymes could not specifically demonstrate GSTM1 expression, the GSTM antibodies were used for Western blotting to see if the antibodies could distinguish polymorphic expression which might represent GSTM1. However, using the GSTM antibodies, no GSTM expression could be demonstrated in lymphocytes of individuals genotyped positive for the GSTM1 gene by PCR.

4.3 Expression of GSTM1 in lung tissue using RT-PCR

Using reverse transcription PCR, the expression of both GSTM1 and GSTM4 enzymes has been demonstrated in lung tissue. Evidence of enzyme expression in lung samples does not, however, conclusively prove that lung cells express GSTs, since inflammatory cells are known to express GSTM1. The inflammatory cell content of disease-free lung tissue should be minimal, nevertheless, the expression of GSTM1 as demonstrated by RT-PCR cannot be attributed exclusively to lung cells.

Local expression of GSTM1 in the lung is unlikely to contribute significantly to disease susceptibility since GSTM1 expression occurs primarily in the liver (Faulder *et al*, 1987). GSTM4 was expressed in every lung sample studied. The substrate specificity of GSTM4 is as yet unknown, however, GSTM1 and GSTM4 are highly homologous (Zhong *et al*, 1993b), which suggests that the 2 enzymes may share similar functions. Recently, Antilla and colleagues (1993) demonstrated expression

of GSTM3 in lung tissue, which showed interindividual variation and enzyme induction in the lungs of smokers. It is possible that both GSTM3 and GSTM4 are important in local lung metabolism of cigarette smoke components. The expression of other glutathione S-transferases which metabolise cigarette smoke components and which demonstrate interindividual variation in the lung may explain the conflicting results obtained in previous studies investigating the role of the GSTM1 polymorphism and susceptibility to lung cancer.

4.4 Analysis of the GSTM1 polymorphism in control and patient populations

4.4.1 Frequency of the GSTM1 polymorphism in the control population

Genotyping of GSTM1 was carried out for 384 anonymous blood samples which represented the control group for comparison in disease association. Of the 384 control samples, 53% were null at the GSTM1 locus, while 47% of individuals were found to have the intact gene. There are many reports in the literature of the frequency of the GSTM1 polymorphism in the general population, some of which are summarized in Table 4.1. As Table 4.1 shows, the reported frequency of the polymorphism is highly variable, and ranges from 41 to 65 percent of populations being null for the GSTM1 gene. Some of this variation can be accounted for by racial differences in frequency of the polymorphism, a phenomenon known to occur in polymorphisms of xenobiotic metabolising enzymes (Cosma *et al*, 1993; Evans,

1989), however, percentages of null individuals are variable even within the same population.

In the UK, for example, the percentage of individuals null for GSTM1 ranges from 41 to 61 percent. Most studies in the UK have generated control population values of approximately 40 percent (Strange *et al*, 1991), with a single Scottish study finding 61% of the population being null for GSTM1 (Strange *et al*, 1985). A finding of 53% null individuals in the Scottish control group studied in this thesis is therefore higher than would be expected from the literature for a UK control group, but is lower than the frequency of 61% for GSTM1 deletion reported by Strange and colleagues for a Scottish population (1985). However, Strange and colleagues genotyped only 52 individuals, hence this study might not fully represent the level of GSTM1 deletion in the Scottish population. Indeed, the size of control groups used in most UK studies of GSTM1 deletion have been too small to generate frequencies representative of the UK population.

While the frequency of GSTM1 deletion in the Scottish population obtained in this thesis does not correlate with that reported by Zhong and colleagues (1991) which used a UK control group numbering 225, it is consistent with the control frequencies reported by Brockmöller and colleagues (1993) and Alexandrie and colleagues (1994) in their large studies of European populations which number over 300 control samples. Large studies by Van Poppel and colleagues (1992) and Kihara and colleagues (1994) are also in disagreement with the data reported here. The Kihara

study concerned Japanese individuals, therefore the frequency of GSTM1 deletion may differ from that of the UK population as a result of racial differences in genetic frequency, as has been reported for other polymorphic xenobiotic metabolising enzymes (Cosma *et al*, 1993). However, the European study by Van Poppel was also inconsistent with this study, but perhaps the use of only males in the control group has produced an unrepresentative control group.

The frequency of GSTM1 deletion in the general population is of primary importance when attempting to ascertain whether the polymorphism is involved in susceptibility to disease. Several studies of the GSTM1 polymorphism and disease susceptibility in the British population have generated frequencies of GSTM1 deletion in groups of patients which have proved significantly different from controls. However, as has been discussed the control frequencies of GSTM1 deletion used in many of these studies may have been too low as a result of insufficient sampling of the population. If control levels of GSTM1 deletion are too low, it is not surprising that significant differences between disease populations and these control groups can be identified.

If the frequency of GSTM1 deletion for the control group obtained in this thesis was used for comparison with disease group frequencies in some other UK studies, the disease association reported would no longer be significant. Fryer and colleagues (1993a) for example, obtained a frequency of 58% GSTM1 deletion in 195 patients with pituitary adenoma, which was significantly different to the control group (n=89), where 44% of individuals were null at the GSTM1 locus. If the frequency of

GSTM1 deletion in pituitary adenoma patients is compared with the frequency of 53% obtained in this thesis for controls (n=384), the comparison between groups is no longer significant (χ^2=1.088, p=0.297). Furthermore, Fryer and colleagues demonstrated a highly significant difference between GSTM1 deletion in 54 patients with prolactinomas and their control group numbering 89 (χ2=6.10, p=<0.05, increased relative risk with GSTM1 null genotype of 2.56). However, if the frequency of GSTM1 deletion in patients with prolactinomas is compared to the control group frequency obtained in this thesis, no significant difference is observed (χ2=3.374, p=0.067).

Fryer and colleagues also used a control group assembled from several published papers on the frequency of GSTM1 deletion for comparison with their prolactinoma data. While this brought the overall number of subjects in the control group to over 500, of whom 50% were null at the GSTM1 locus, of the studies which comprised this combined control group, all genotyped less than one hundred individuals, with the exception of the study of Kihara and colleagues which investigated the genotype of 166 Japanese individuals. Using a control group numbering over 500 individuals would seem to be rigorous in attempting to reduce the error which might be incurred in selection of a control group, however, if this control group is composed from smaller studies included together, the degree of error entailed by using small control groups is still present within the calculation of the large control group frequency. This large control group used by Fryer and colleagues was also employed by a second group of investigators who demonstrated a significant difference in the

frequency of GSTM1 deletion between these controls and a group of patients with adenocarcinoma of the stomach and colon (Strange *et al*, 1991).

Control group nationality	Total subjects genotyped	% population null for GSTM1	Type of assay used for assessment*	Reference
Caucasian	40	65	P	Board *et al*, 1981
Chinese	96	58	P	Board *et al*, 1981
Dutch	221	45	P	Van Poppel *et al*, 1992.
French	56	43	P	Laisney *et al*, 1984
German	355	51	G,F,P	Brockmöller *et al*, 1993
Indian	43	35	P	Board *et al*, 1981
Japanese	201	45	G	Kihara *et al*, 1994
Japanese	168	48	P	Harada *et al*, 1987
English	49	41	P	Strange *et al*, 1984
UK	225	42	G,P	Zhong *et al*, 1991
UK	89	44	G,P	Fryer *et al*, 1993a
UK	69	45	G,P	Davies *et al*, 1993
UK	58	47	G	Daly *et al*, 1993
Scottish	52	61	P	Strange *et al*, 1985
Swedish	329	53	G,F	Alexandrie *et al*, 1994
USA	192	42	F	Seidegard *et al*, 1990
USA (Blacks)	180	35	G	Bell *et al*, 1993
USA (Whites)	497	49	G	Bell *et al*, 1993
USA	38	47	G	Shields *et al*, 1993

Table 4.1 Published frequencies of the GSTM1 polymorphism in control populations. * abbreviations for assay type are G- genetic, F- functional, P- protein.

4.4.2 GSTM1 deletion and susceptibility to emphysema and lung cancer in biopsy and autopsy cases

Deletion of the GSTM1 gene is associated with susceptibility to emphysema coexistant with lung cancer, but not to cancer with no concomitant emphysema. Significant differences between the frequency of the polymorphism in the control group, and all patients with emphysema were obtained. GSTM1 enzyme has a very broad substrate specificity which includes oxygen derived reactive species, many of which are present in cigarette smoke (Ketterer *et al*, 1988). It is therefore perhaps not surprising that a lack of expression of this enzyme might affect susceptibility to a disease which results from cigarette smoke exposure. While GSTM1 is predominantly a liver enzyme, it is known that much cigarette smoke metabolism and detoxification occurs in the liver and that cigarette smoke related disease states affect many organs and not just the lungs.

The pathogenesis of centriacinar and panacinar emphysemas is generally thought to be distinct (Saetta *et al*, 1994b). Association with GSTM1 deletion might therefore be expected to occur more strongly with one type of emphysema, hence samples were analysed according to their pattern of emphysema. No association was found to occur between GSTM1 deletion and either centriacinar or panacinar emphysema alone. However, in cases with both patterns of disease, a small association was demonstrated, indicating that susceptibility to the development of both centriacinar and panacinar emphysema may result in indviduals with deletion of GSTM1.

A link between GSTM1 deletion and susceptibililty to 2 types of emphysema when present in the same lung sample, but not when present separately, suggests that GSTM1 deletion has a role in the pathogenesis of both forms of emphysema. Since all cases studied were of mild to moderate severity, it may be argued that the level of damage required to give both centriacinar and panacinar emphysemas together is greater than that producing moderate or mild centriacinar or panacinar alone. GSTM1 deletion is likely to have a subtle effect on disease susceptibility, therefore only with greater amounts of damage would the influence of this polymorphism be detected.

Since GSTM1 is expressed at high levels in the liver, it was anticipated that the enzyme polymorphism would have an affect on disease susceptibility in a systemic manner. Panacinar emphysema is associated with deficiency in the systemic protein α1-antiprotease (Guenter *et al*, 1968), therefore GSTM1 deletion might be expected to increase susceptibility to this type of emphysema. However, GSTM1 deletion was not found to be associated with panacinar emphysema alone, but was associated with the presence of both panacinar and centriacinar emphysemas in the same lung sample. This implies that GSTM1 is involved in protection against tissue damage at both the systemic and local level, and therefore that GSTM1 is expressed in both lung and liver.

The increased relative risk demonstrated for the association between GSTM1 polymorphism and susceptibility to emphysema is relatively small. It is not

surprising that the increased risk identified is small since GSTM1 is only one of a cohort of xenobiotic metabolizing enzymes, many of which have overlapping substrate specificities and may themselves be polymorphic. Although small, the increased risk demonstrated is likely to account for a significant number of cases of emphysema, since emphysema is a very common disease.

No association was found between GSTM1 polymorphism and emphysema in autopsy cases. This result is surprising, particularly since an association was found between GSTM1 deletion and emphysema in biopsy cases. However, the emphysema found in autopsy cases is generally more severe than that of biopsy samples since autopsies are often performed on individuals in whom emphysema has contributed significantly to cause of death, whereas biopsies are obtained from individuals fit enough to survive surgery.

GSTM1 is one xenobiotic metabolising enzyme among a cohort of enzymes affording protection in the lung, therefore the effect of any deficiency is likely to be marginal, particularly since many other metabolising enzymes have similar substrate specificities. Furthermore, if the lung tissue is exposed to very great amounts of xenobiotic insult, the effects of deficiency in a single enzyme are unlikely to be significant since the xenobiotic metabolising system will be overwhelmed. The small increased relative risk for moderate to mild emphysema identified in individuals null for GSTM1 indicates that xenobiotic metabolising enzymes do have a role in influencing the development of emphysema. Indeed, there are other

polymorphic xenobiotic metabolising enzymes which are possibly more influential in increasing susceptibility to emphysema. Recent evidence suggests that the marginal effect of the GSTM1 polymorphism is not apparent when more serious defects such as the epoxide hydrolase 'slow' polymorphism are present (Smith CAD, personal communication). Conceivably, severe emphysema results when several crucial influencing factors combine to increase susceptibility to injury.

Deletion of the GSTM1 gene was not associated with microscopically assessed emphysema. However the GSTM1 polymorphism was shown to be associated with 2 different types of macroscopic emphysema where there was concomitant lung cancer, which suggests that the enzyme may influence disease at an early stage of damage. Therefore it is perhaps surprising that the GSTM1 polymorphism is not associated with early emphysema, which is only detectable by microscope. This contradiction suggests that GSTM1 deletion does not influence very mild disease since the loss of enzyme function can be compensated for within the protective system of metabolising enzymes. As more damage occurs within the lung, as a result of increased exposure to toxicity, the loss of this enzyme becomes more influential as the resources of protective enzymes are stretched. Finally, with very great amounts of damage, caused by overwhelming levels of toxic insult, deficiency of a single enzyme has little effect on the disease process. This hypothesis may explain why GSTM1 deficiency does not influence susceptibility to either very early emphysema, as assessed microscopically, or to very severe emphysema, such as that seen in

autopsy cases, but the polymorphism is associated with the mild to moderate emphysema found in biopsy lung samples.

For this theory to be correct, deficiency of GSTM1 must influence the disease process in a dose dependant manner. Association between the GSTM1 polymorphism and microscopic emphysema was investigated particlularly because some cigarette smokers are at an increased risk of developing this type of emphysema in a manner which is not dependant on their exposure to cigarette smoke (Gillooly & Lamb, 1993a). If GSTM1 deficiency does influence disease susceptibility in the dose-dependant manner outlined above it is perhaps not surprising that this enzyme polymorphism is not associated with microscopic emphysema.

4.4.3 Lack of association between GSTM1 and lung cancer

Since emphysema is a lung disease which results from exposure to cigarette smoke, it is often found concomitant with lung cancer. The majority of cases studied contained both emphysema and lung cancer. There is evidence that GSTM1 deletion might be involved in increasing susceptibility to lung cancer (Seidegard *et al*, 1986; Howie *et al*, 1990a; Seidegard *et al*, 1990; Zhong *et al*, 1991; Hirvonen *et al*, 1993; Kihara *et al*, 1994; Alexandrie *et al*, 1994). It is therefore possible that an

association between polymorphism of GSTM1 and emphysema may actually derive from an underlying association with lung cancer.

However, no significant association could be demonstrated between the GSTM1 polymorphism and susceptibility to lung cancer either in a heterogeneous group of lung cancer cases, or when the total lung cancer cases genotyped for GSTM1 were subdivided according to type of disease.

The evidence linking the GSTM1 polymorphism with susceptibility to lung cancer is confusing. Susceptibility to lung cancer of all types has been reported by several groups (Seidegard *et al*, 1986; Howie *et al*, 1990a;, Seidegard *et al*, 1990), but other investigators have failed to demonstrate similar findings (Brockmöller *et al*, 1993; Zhong *et al*, 1991). When GSTM1 deletion has been investigated for conferring susceptibility to different types of lung cancer the results have again been conflicting. GSTM1 deletion has been associated with susceptibility to squamous carcinoma of the lung by several groups (Zhong *et al*, 1991; Hirvonen *et al*, 1993; Kihara *et al*, 1994; Alexandrie *et al*, 1994). The GSTM1 polymorphism was associated with susceptibility to adenocarcinoma by Seidegard and colleagues in 1990, but Zhong and colleagues (1991) found a negative correlation between GSTM1 deletion and adenocarcinoma. Small cell carcinoma has been linked to the GSTM1 polymorphism by both Kihara and colleagues (1994) and Alexandrie and colleagues (1994).

While this evidence does seem to point to a role for GSTM1 deletion in increasing susceptibility to lung cancer it is not possible with the present information to understand exactly what role this might be. It is clear that several factors are involved in moderating the effects of the polymorphism. Differences in racial expression may explain some of the conflicting results. Polymorphic xenobiotic metabolising enzyme expression is known to vary between races, and the reports are derived from different countries although most have investigated Caucasian populations. Sex differences may also modulate the effects of GSTM1. While the polymorphism has not been reported to be disproportionately expressed in either sex, one study demonstrated an association between GSTM1 deletion and squamous carcinoma in women, but not in men (Alexandrie *et al*, 1994). Furthermore, Alexandrie and colleagues found that while there was an association between GSTM1 polymorphism and small cell carcinoma, this link was significantly related to the age of the patients studied.

While investigating the GSTM1 polymorphism in a large group of lung cancer patients was outwith the scope of this thesis, a small group of heterogeneous lung cancer cases was studied. Forty-six lung cancer samples that contained no evidence of emphysema were genotyped and analysed in comparison with the control group. No association was demonstrated to occur between deletion of GSTM1 and lung cancer. This lack of association with lung cancer suggests that the association demonstrated for groups of patients with both emphysema and lung cancer may be a

reflection of effects of the polymorphism on emphysema, and not on concomitant lung cancer.

An association between the GSTM1 polymorphism and susceptibility to emphysema has important implications for research into lung cancer susceptibility. Just as it is possible that an association demonstrated between GSTM1 deletion and emphysema may reflect an underlying association with lung cancer, the reverse situation is also possible, where a lack of GSTM1 may be implicated in lung cancer when the association is merely the result of concomitant emphysema in the samples studied. This possibility might help explain the disparity in the association of lung cancer and GSTM1 deletion in the literature.

4.4.4 GSTM1 deletion is not associated with clinically defined COPD

The clinical manifestation of emphysema is chronic obstructive pulmonary disease, which represents a heterogeneous group of lung diseases that cause obstruction of the airways and difficulty in breathing. A group of patients attending a chest clinic for treatment of chronic obstructive pulmonary disease were not found to have any significant differences in the proportion of GSTM1 deletion when compared to the control population. This result is perhaps surprising. COPD is a heterogeneous disease, nevertheless some propensity for developing COPD might be expected to occur when GSTM1 is absent.

It is possible that no association between COPD and the GSTM1 polymorphism was demonstrable because in individuals attending COPD clinics lung tissue destruction is likely to be extensive, and of much greater severity than that seen in the lungs of biopsy samples in which an association with GSTM1 has been demonstrated. Biopsy samples are only obtained from patients who are fit for surgery, therefore the biopsy cases studied have been selected for a milder level of disease than would generally be found in a chest clinic. Perhaps in end-stage lung disease so much exposure to toxic compounds has generated such great amounts of destruction that the singular effects of one polymorphism cannot be detected.

Another possible explanation for the lack of association between GSTM1 deletion and COPD is that the relationship between emphysema and COPD as defined by decreased lung function is not necessarily direct. While increased airspace size in the parenchyma may reduce elastic recoil thereby affecting lung function, severe emphysematous lesions are not thought to contribute significantly to the pressure/volume relationship of the lung nor to a decreased expiration volume of the lung, because very little change in the volume of severe lesions occurs with lung deflation (Paré & Hogg, 1995).

The finding of a lack of association between GSTM1 deletion and COPD has also been demonstrated by Alexandrie and colleagues, who used a group of patients with chronic obstructive pulmonary disease as a control population (Alexandrie *et al*, 1994).

4.4.5 GSTM1 polymorphism and susceptibility to disease

GSTM1 is associated with both emphysema and lung cancer, and to the development of both centriacinar and panacinar emphysema, which may be two different diseases with distinct causes (Saetta *et al*, 1994b). This suggests that an individual lacking GSTM1 may be more susceptible to lung disease caused by cigarette smoking.

Evidence that GSTM1 is expressed in lung tissue suggests that individuals with the deletion polymorphism of GSTM1 may have increased susceptibility both at the local and systemic level, reflecting a lower threshold to any injury involving oxygen reactive species or GSTM1 substrates, including environmental pollutants, and not just carcinogenic cigarette smoke components. This is in keeping with the association between GSTM1 deletion and a wide variety of apparently unrelated disease states (Strange *et al*, 1991; Heagerty *et al*, 1994; Zhong *et al*, 1991; Seidegard *et al*, 1990).

The GSTM1 polymorphism may also affect disease susceptibility by influencing the expression of other enyzmes in the lung. Recent work by Nakajima and colleagues (1995) has shown that expression of GSTM1 influences the expression of GSTM3 and possibly GSTM2. Where individuals were found to be null for GSTM1, significantly lower levels of GSTM3 were observed compared to levels of GSTM3 in individuals with the intact GSTM1 gene. Furthermore, a second GST, which the investigators ascribed to GSTM2 was only observed in lung samples which

demonstrated GSTM1 expression. Antilla and colleagues (1995) also showed reduced expression of GSTM3 in the parenchyma of GSTM1 null individuals who were current smokers or recent ex-smokers. The expression of GSTM3 was not affected by GSTM1 genotype in the bronchial epithelium, which suggests that the genotype and expression of these enyzmes may have further effects on the differential metabolism of reactive species in the lung. Since GSTM3 has been shown to have catalytic activity towards electrophiles (Hussey & Hayes, 1993) any effect on expression of this enzyme as a consequence of the GSTM1 polymorphism may therefore have a subsequent effect on the levels of reactive species, increasing susceptibility to disease.

Support for the association of GSTM1 status and disease susceptibility comes from *in vitro* studies of leukocytes which demonstrate that cells from GSTM1-null individuals are more prone to epoxide-induced DNA injury and sister chromatid exchange (van Poppel *et al*, 1992; Wiencke *et al*, 1990). GSTM1 deletion has also been linked with increased DNA-adduct levels in lung tissue (Shields *et al*, 1993; Ketterer *et al*, 1992; Grinberg-Funes *et al*, 1994). For this reason the determination of GSTM1 genotype is important in considering individual susceptibility to xenobiotic inhalation or cigarette smoke. This is equally relevant to metabolism occurring in the lung as well as other organs, including the liver where there is high expression of the enzyme (Faulder *et al*, 1987).

4.5.1 Allele frequencies of the CYP1A1 exon 7 polymorphism in the Scottish population

In the control population the CYP1A1 exon 7 polymorphism was found to be present in 1% of individuals as homozygotes for the rare valine allele, 12% of controls were heterozygotes, while 87% of the population were homozygous for the isoleucine allele of CYP1A1. These results reflect an allele frequency of 0.93 for the isoleucine allele and 0.07 for the valine variant of CYP1A1.

The CYP1A1 allele frequencies in the control population are not significantly different to those presented by other groups in the literature. While the frequency of the valine rare allele has been reported in higher numbers in the Japanese (Hayashi *et al*, 1992), with a frequency of 0.20, this probably reflects ethnic differences in the expression of this polymorphism. European studies by Alexandrie and colleagues (1994) and Hirvonen and colleagues (1992) identified CYP1A1 valine allele frequencies of 0.03 and 0.05 respectively. A further study investigated the frequency of the valine variant in Japanese, Hawaiian and Caucasian populations and reported frequencies of 0.19, 0.07 and 0.00 respectively (Sivaraman *et al*, 1994). The control

population numbers studied in the Japanese and European reports were large, numbering well over 200 individuals in all reports, whereas the study of Sivaraman and colleagues (1994) used small control groups. The valine allele frequency is therefore slightly higher in the Scottish population than might have been expected from other studies of Caucasian populations.

4.5.2 Polymorphism of CYP1A1 and association with emphysema and coexistent lung cancer

The isoleucine to valine polymorphism of CYP1A1 has been shown to be associated with macroscopic emphysema. A significant difference was demonstrated to exist between the control group and patients with emphysema. No association could be demonstrated between CYP1A1 polymorphism and either centriacinar or panacinar emphysema alone nor to both patterns of disease together. The group of patients studied suffered from lung cancer with or without concomitant emphysema. Association with the CYP1A1 polymorphism did not occur in cases with no evidence of macroscopic emphysema, nor in the total group of lung cancer patients including those with and without emphysema. CYP1A1 association only occurred in the group of cancer cases with concomitant emphysema.

While no statistically significant association could be demonstrated between a specific pattern of emphysema and the CYP1A1 polymorphism, a trend towards increased numbers of heterozygotes for the polymorphism was detected in patients with centriacinar emphysema singly and in patients with both centriacinar and panacinar emphysemas together.

The finding that the CYP1A1 polymorphism has an affect on susceptibility to macroscopic emphysema, with a particular slant towards a propensity for the development of centriacinar emphysema is not unexpected. McLemore and colleagues have shown that CYP1A1 is expressed in lung tissue following cigarette smoke exposure (McLemore *et al*, 1990). When smoke enters the lungs, it travels through the airways until it reaches the respiratory bronchioles, where airflow ceases (Harley, 1988). It is in this region of the lung that most direct damage will occur as a result of cigarette smoke exposure, since particles contained in the smoke bounce into the airway walls and are absorbed (Harley, 1988). In this manner, polycyclic aromatic hydrocarbons contained in cigarette smoke will be absorbed into lung tissue primarily at the respiratory bronchioles, where they will induce the expression of CYP1A1. If the CYP1A1 gene induced contains the polymorphic valine allele, the resulting enzyme will be more efficient at activating polycyclic aromatic hydrocarbons to reactive intermediates (Hayashi *et al*, 1991b, quoted in Kawajiri *et al*, 1993), which can then contribute to tissue destruction. Thus, the area of the lung which might be expected to be affected particularly by polymorphism of the

CYP1A1 gene is the respiratory bronchioles, which is the region of the lung involved in centriacinar emphysema (Lamb, 1995).

A lack of association between the CYP1A1 polymorpism and panacinar emphysema alone is also not a surprising result. Panacinar emphysema has been shown to result in individuals suffering from α1-antiprotease deficiency (Laurell & Eriksson, 1963), indicating that the damage which results in this disease derives from a primarily systemic source. Since CYP1A1 is expressed locally in lung tissue, and is an extrahepatic enzyme, cigarette smoke metabolism by the enzyme will occur locally and would therefore not be likely to have an effect on systemic disease.

The association between CYP1A1 polymorphism and macroscopic emphysema was obtained only in cases of emphysema with concurrent lung cancer. This finding implies that any increased susceptibility to lung disease conferred by the CYP1A1 polymorphism is an early event, occurring at a stage before the injury response pathway leading to cell death and inflammation has diverged from that leading to mutagenesis and eventual tumour progression. This is in keeping with the likely position of CYP1A1 in the pathway of cigarette smoke metabolism that is, it is proximate to the insult by nature of its expression in lung parenchyma and its role in phase I metabolism.

While polymorphism of CYP1A1 is associated with emphysema and lung cancer the increased relative risk found is rather small, and is only significant when both diseases are present. However, emphysema and lung cancer are extremely prevalent diseases

hence even a slight increase in disease susceptibility conferred by this polymorphism may account for a large proportion of cases.

The isoleucine to valine polymorphism of CYP1A1 was also found to be associated with microscopic emphysema, which represents early disease, with a relatively high odds ratio and confidence intervals. This finding correlates well with both the early role of CYP1A1 in Phase I metabolism, and the induction of enzyme expression in lung tissue by cigarette smoke components, and provides good evidence that CYP1A1 polymorphism is involved in the development of early disease resulting from exposure to cigarette smoke and other toxic xenobiotics. Furthermore, studies by Gillooly & Lamb (1993c) have shown that there is a population of smokers who have increased susceptibility to microscopic emphysema which is not dependant on their level of exposure to cigarette smoke. Conceivably the CYP1A1 polymorphism may account for this susceptibility since the association between the polymorphism and microscopic emphysema is very strong ($p=0.001$).

4.5.3 Polymorphism of CYP1A1 is not associated with lung cancer

Genotyping of CYP1A1 was carried out on a heterogeneous lung cancer patient group in whom the presence of emphysema had been excluded. This enabled investigation into whether or not the association found in emphysema cases was due to a real relationship with emphysema, or was the result of an association with concomitant lung cancer, which was present in a high proportion of the cases studied.

No association could be demonstrated between the CYP1A1 polymorphism and susceptibility to lung cancer.

Previous studies investigating CYP1A1 and lung cancer have provided conflicting data. Whereas in Japanese populations CYP1A1 conferred a 3-fold risk of lung cancer (Nakachi *et al*, 1991; Kawajiri *et al*, 1993), similar studies of Scandinavian populations have failed to find any association (Tefre *et al*, 1991; Hirvonen *et al*, 1992; Alexandrie *et al*, 1994).

Confounding factors have recently been shown to be of importance in studies of association between CYP1A1 polymorphism and lung cancer. Histological type of tumour has been shown to be significant, with certain types of tumour more likely to be influenced by CYP1A1 genotype. For example, Nakachi and colleagues (1995) have shown that in addition to the association between CYP1A1 polymorphism and lung squamous cell carcinomas which they had previously identified (Nakachi *et al*, 1991; Nakachi *et al*, 1993), there is also an association between this enzyme polymorphism and adenocarcinoma of the lung, but this association is related to grade of tumour differentiation. Level of cigarette smoke exposure has also been shown to affect the association between CYP1A1 genotype and lung cancer. Okada and colleagues (1994), have demonstrated that squamous cell carcinoma is significantly associated with genotype C of the CYP1A1 *MspI* polymorphism, especially in light smokers ($p<0.001$). Lymph node or distant metastasis was also found to be more frequent in individuals with squamous carcinomas, especially

among light smokers, with genotype C ($p<0.05$). Association linked to cigarette smoke exposure was also demonstrated by Sugimura and colleagues (1995), who demonstrated that the Ile-Val polymorphism of CYP1A1 was associated with squamous carcinoma, particularly in light smokers.

Perhaps in using a small, heterogeneous group of lung cancer cases, association may not have been detected through not analysing confounding factors such as tumour types, cigarette smoke exposure, age, sex and metastatic frequency in the tumours studied. However, no study to date has considered the possibility that CYP1A1 may confer susceptibility not just to lung cancer but also to other forms of lung disease, such as emphysema, and that association with other diseases might influence or even explain the conflicting associations seen between CYP1A1 polymorphism and lung cancer.

4.5.4 Lack of association between CYP1A1 polymorphism and COPD

No association was found to exist between polymorphism of the CYP1A1 gene and susceptibility to chronic obstructive pulmonary disease. This result is perhaps surprising since COPD is the clinical manifestation of emphysema, which has been shown to be associated with CYP1A1 polymorphism. One other study has investigated the CYP1A1 polymorphism in a group of chronic obstructive pulmonary disease patients (Alexandrie *et al*, 1994). No difference in the distribution of

CYP1A1 alleles was found between COPD patients and healthy controls in this study.

It is likely that the explanation for a lack of association with COPD, even though an association has been demonstrated with emphysema, is a consequence primarily of the increased severity of emphysema in COPD and perhaps also of the heterogeneous nature of COPD. Clinical cases of chronic obstructive airways disease do not represent only emphysema, but also include chronic obstructive airways disease secondary to chronic bronchitis. The cases with both lung cancer and emphysema which were derived from biopsy samples had mild or moderate emphysema relative to the clinical group with chronic obstructive airways disease which represented very severe disease. Perhaps, the CYP1A1 polymorphism contributes little towards susceptibility to severe, non-neoplastic lung injury. Conceivably, in severe emphysema such great amounts of exposure to toxins has generated so much damage that the lung defense represented by xenobiotic metabolising enyzmes is overwhelmed, and polymorphic expression of these enzymes is of negligible effect.

4.5.5 CYP1A1 polymorphism and susceptibility to disease

The CYP1A1 polymorphism was associated with emphysema and concomitant lung cancer. Thus polymorphism of CYP1A1 is involved in the mechanisms which result in both tumorigenesis and the tissue destruction seen in emphysema. This implies that the polymorphism has a general role in the protection or damage of tissues against disease.

A further speculation can be made following the demonstration that the CYP1A1 polymorphism is associated with microscopic emphysema which represents very early disease, and with mild to moderate emphysema only in the presence of concomitant lung cancer. This finding suggests that the effect of the polymorphism manifests at a very early stage of disease progression in the lung following cigarette smoke exposure. This is perhaps not surprising since CYP1A1 is involved in Phase I metabolism and therefore represents an early defense mechanism preventing tissue destruction (Gibson & Skett, 1986). The lack of association between the CYP1A1 polymorphism and COPD, an end-stage lung disease, corroborates this theory.

4.6 Lung cancer and polymorphisms of GSTM1 and CYP1A1

Previous studies investigating polymorphisms of GSTM1 and CYP1A1 have not produced consistent evidence to support their involvement in conferring susceptibility to lung cancer. In the case of GSTM1 deletion, both adenocarcinoma, squamous cell carcinoma and small cell carcinoma have been implicated as associated diseases in separate studies (Seidegard *et al*, 1990; Zhong *et al*, 1991; Hirvonen *et al*, 1993; Alexandrie *et al*, 1994). Polymorphism of the CYP1A1 gene has been shown to be associated with susceptibility to lung cancer in Japanese populations (Nakachi *et al*, 1991; Kawajiri *et al*, 1993), however studies on Caucasians have been unable to verify these findings (Tefre *et al*, 1991; Hirvonen *et*

al, 1992; Alexandrie *et al*, 1994). Ethnic differences may account for some of the inconsistencies in the literature, however, they cannot explain the disparity in all cases. In particular, GSTM1 deletion has been shown to be associated with lung cancer generally, and specifically with squamous cell carcinoma, adenocarcinoma and small cell carcinoma but a consistent pattern of disease susceptibility has not yet been detected. It seems unlikely that ethnic differences are the reason for these contrasting reports. This study demonstrates that polymorphisms of GSTM1 and CYP1A1 are involved in susceptibility to emphysema. From the results obtained in this study, it might be speculated that susceptibility to emphysema conferred by polymorphic GSTM1 or CYP1A1 has influenced studies of these polymorphisms in lung cancer. Indeed, there are other polymorphic xenobiotic metabolising enzymes which may also be regarded as candidates for influencing susceptibility to both lung cancer and emphysema.

4.7 Polymorphic xenobiotic metabolising enzymes and susceptibility to disease

Association of the GSTM1 and CYP1A1 polymorphisms with different types of emphysema and cancer suggests that these enzyme polymorphisms have a general role in influencing disease susceptibility. Furthermore, the association between the polymorphisms with different types of emphysema and with different diseases implies that the enzymes have their protective effects at an early stage in the pathway to tissue damage, scarring and tumorigenesis. The finding that neither the GSTM1

nor the CYP1A1 polymorphisms were associated with chronic obstructive pulmonary disease, an end-stage lung disease, provides evidence to substantiate this hypothesis.

Recently, evidence has been presented which indicates that the polymorphisms of GSTM1 and CYP1A1 can increase susceptibility to lung cancer in a synergistic manner (Hayashi *et al*, 1992). The mechanism behind this synergistic increase in susceptibility is presumably that with a more highly inducibile CYP1A1 increased amounts of reactive species will be generated. Where there is a concurrent deletion in GSTM1, the defence against reactive species provided by Phase II metabolism is compromised, leading to increased amounts of damage.

If this phenomenon of synergistic effects on disease susceptibility can occur for polymorphisms of GSTM1 and CYP1A1, it may also result with other polymorphisms in the system of xenobiotic metabolising enzymes. There is considerable evidence to support a general role for these enzymes in influencing susceptibility to cancer, and there have been several reports of xenobiotic metabolising enzymes being involved in the development of non-neoplastic disease including pituitary adenoma (Fryer *et al*, 1993), Parkinson's disease (Smith *et al*, 1992), and chronic liver hepatitis (Harada *et al*, 1987).

Furthermore, in the context of emphysema and lung cancer, there are many other xenobiotic metabolising enzymes which are likely to influence susceptibility to these

diseases by virtue of their expression in the lung and their substrate specifities. Polymorphisms of the xenobiotic metabolising system are likely to influence disease susceptibility through a number of detrimental and beneficial effects mediated by the interacting network of enzymes.

GSTM1 deletion and the Ile-Val^{462} and *MspI* polymorphisms of CYP1A1 have also been associated with increased risk for mutation in the tumour suppressor gene p53 (Ryberg *et al*, 1994; Kawajiri *et al*, 1996). Thus polymorphisms of xenobiotic metabolising enzymes influence disease susceptibility not only through the network of metabolising enzymes but may affect disease processes through their effects on other genes.

The effect of xenobiotic metabolising enzyme polymorphisms on disease susceptibility will be influenced by other mechanisms of disease development. These other mechanisms may be more important than xenobiotic metabolising enzyme polymorphisms in determining the likelihood of cigarette smoke injury resulting in predominantly genotoxic or cytotoxic injury. Thus certain protective mechanisms such as other enzymes of the glutathione dependant system, epoxide hydrolase, and differences in proteolytic enzymes or antiproteases may reduce the likelihood of cell damage caused by lipid or protein peroxidation leading to emphysema. Conversely, differences in DNA repair and other genes involved in recognising and eliminating DNA damage may be very important in determining whether or not tumorigenesis can

occur. These mechanisms are likely to differ between individuals and consistent with this is the presence of known polymorphisms/ mutations, for example in the p53 gene and in the DNA mismatch repair system (Service, 1994; Papadopoulos *et al*, 1994; Marx, 1993). These polymorphisms or mutations may contribute to interindividual differences in the capacity to eliminate DNA damage and therefore prevent mutations leading to tumourigenesis.

Only in cases with no bias towards, or away from, non-neoplastic or neoplastic lung disease would polymorphism of GSTM1 or CYP1A1 be expected to have a detectable effect on the susceptibility to disease rather than simply a more subtle underlying modification of mechanisms and responses to injury.

4.8 Genetic susceptbility to Emphysema

This is the first time that investigation into susceptibility to emphysema has focused on a genetic basis for the disease, other than the well-characterised susceptibility which is governed by deficiency in α1-antiprotease. Polymorphism in both the GSTM1 and CYP1A1 genes have been shown to be associated with emphysema, and this evidence opens up the way for further research into whether other polymorphic xenobiotic metabolising enzymes might have a similar role in increasing the risk of

an individual to develop emphysema. Within the system of xenobiotic metabolising enzymes, there are many known polymorphisms, some of which have been characterized at the genetic level, and some of which have been identified through pharmacogenetic variance (Gibson & Skett, 1986). Since many of these polymorphic enzymes will have substrate specificities which include components of cigarette smoke, this field of study represents an ideal opportunity for further research.

Of particular interest might be the enzyme epoxide hydrolase, which is known to be involved in benzo[a]pyrene metabolism (Gibson & Skett, 1986), and is polymorphic (Hassett *et al*, 1994). The cytochromes P450 2F1, 2D6, and 3A4 might conceivably have roles in increasing susceptibility to emphysema. CYP2F1 is thought to be polymorphic, it is expressed in lung tissue, and it metabolises cigarette smoke components (Nhambuto *et al*, 1989). CYP3A4 is expressed in lung and liver tissue, and expression in the liver has been shown to be highly variable (McManus *et al*, 1990; Shimada *et al*, 1989). Furthermore, CYP3A4 substrates include compounds present in cigarette smoke. CYP2D6 is polymorphic, and the polymorphism has been associated with increased susceptibility to breast, bladder, and lung cancer, leukaemia and Parkinson's disease. However, the association between CYP2D6 and susceptibility to breast, lung, and bladder cancer has not been categorically proven since genotype studies do not affirm the original association identified with phenotyping assays (Smith, 1994).

4.9 Identification of a novel CYP1A1 variant

While sequencing a sample which had previously been identified as homozygous for the valine allele of the CYP1A1 gene, it was discovered that the sample did not in fact have the valine allele but had an isoleucine at amino acid residue 462, and was subject to a different polymorphism. This novel CYP1A1 sequence contains an asparagine residue instead of a threonine at amino acid position 461 of the CYP1A1 gene. Hayashi and colleagues demonstrated that the isoleucine to valine substitution had an affect on the ability of the enzyme to activate polycyclic aromatic hydrocarbons in yeast (1991b, quoted in Kawajiri *et al*, 1993). From this finding, Kawajiri and colleagues speculated that the effect of the mutation might result from disruption of a haem-binding thiolate ligand, a cysteine residue present 5 residues upstream of the site of the mutation. Since the novel threonine to asparagine substitution identified in this study is even closer to the crucial cysteine residue which is required for haem-binding of CYP1A1, it is possible that this polymorphism also affects enzyme function, and may even be more disruptive of function than the previously described isoleucine to valine substitution.

Previous studies concerning the CYP1A1 polymorphism have utilised 2 PCR strategies to detect the polymorphism. Most investigators have used the allele specific PCR described by Hayashi and colleagues (1991a). This method employs PCR primers specific for either of the 2 possible alleles resulting from the isoleucine/valine polymorphism along with an upstream primer which will amplify

either of the polymorphic variants. Genotype is determined by 2 separate PCRs using each of the specific primers with the common upstream primer. The PCR strategy employed by Hayashi and colleagues, and other researchers who subsequently used this method, would not have detected the novel polymorphism of CYP1A1 described here. The allele specific primers designed by Hayashi and colleagues do not cover the site of the threonine to asparagine substitution, hence either of these alleles would be amplified using this technique.

The other strategy used to detect the CYP1A1 Ile/Val polymorphism is similar to the method described in this thesis, which has also coincidentally been used by Shields and and colleagues (1993). This strategy engineers a downstream primer to create an *NcoI* restriction enzyme site in the Ile^{462} variant of the gene, which is destroyed in the Val^{462} variant. While this strategy does detect the threonine to asparagine polymorphism, since this base change also results in destruction of the *NcoI* site, this polymorphism can only be distinguished from the substitution causing the Ile/Val polymorphism by cloning and sequencing the PCR products.

The study by Shields and colleagues (1993) using a similar PCR strategy to identify the Ile-Val polymorphism was only used to genotype 38 individuals. Of these 38, 32 were homozygous for the Ile^{462} allele, 2 were heterozygotes, and 4 had only the Val^{462} allele of CYP1A1. While the number of individuals found to be homozyous for the valine allele does seem higher than might be expected for a Caucasian population, the number of individuals genotyped is perhaps too small to allow

specultation that the higher frequencies obtained by Shields and colleagues can be ascribed to the novel Thr-Asn polymorphism.

Only sequencing cloned DNA from an individual with the asparagine variant of the CYP1A1 gene would detect this novel polymorphism. Hayashi and colleagues did sequence genomic DNA from individuals of each genotype for the Ile/Val polymorphism (1991a), but none of the investigators who have subsequently studied this polymorphism present sequencing evidence to validate their PCR results. In the original publication of the CYP1A1 cDNA gene sequence, sequencing of each gene fragment was carried out for both strands at least once, and commonly the fragments were sequenced between 5 and 20 times (Jaiswal *et al*, 1985). Therefore, it is possible that the CYP1A1 polymorphism identified in this thesis has not been previously detected because the gene has only been sequenced in this region a limited number of times.

To provide some preliminary information on the frequency of this novel variant of the CYP1A1 gene in the population, the genotype of 27 indidviduals was ascertained by using a novel PCR and restriction enzyme assay which allowed detection of the asparagine variant. Of these 27, 26 had previously been genotyped as heterozygotes, and of these, 14 were found to have the asparagine polymorphism while 12 were found to be heterozygotes for the isoleucine/valine polymorphism. The other individual genotyped had previously been shown to be a homozygote for the Val^{462}

variant, however, restriction enzyme analysis indicated that this person was actually homozygous for the asparagine polymorphism of CYP1A1.

This data is preliminary, but it does imply that the threonine to asparagine polymorphism of CYP1A1 is common in the Scottish population, and may even be more common than the Val^{462} variant of CYP1A1 previously characterised. However since only 27 individuals have been genotyped, the frequency of the polymorphism requires confirmation. The high frequency of the novel polymorphism in this subset of the control group has implications for the disease association demonstrated in this thesis, however it is difficult with the limited information available to predict whether or not this polymorphism will effect enzyme function and disease susceptibility. Certainly the increased level of exon7 polymorphism in the general population demonstrated in this thesis as compared to other studies is likely to be a reflection of detection of the novel polymorphism as well as the Ile-Val^{462} polymorphism. Whether or not the increased frequency of exon 7 polymorphism in individuals with emphysema and concomitant lung cancer is the result of disproportionately high Ile-Val^{462} or Thr-Asn^{461} variants remains to be shown.

4.10 Opportunities for further research

As well as demonstrating a role for xenobiotic metabolising enzymes in modifying susceptibility to emphysema, the work presented in this thesis has identified several other aspects of emphysema and xenobiotic metabolising enzymes which merit further study.

4.10.1 The novel CYP1A1 polymorphism

A novel variant of the CYP1A1 gene has been identified. This polymorphism results in amino acid replacement close to the cysteine residue which is critical for haem-binding of the CYP1A1 protein, and may therefore influence the function of the protein. The previously characterised isoleucine to valine substitution in the same region of the CYP1A1 gene has been shown to result in increased activity of the protein (Hayashi *et al*, 1991b, quoted in Kawajiri, *et al*, 1993), therefore this novel variant may have a similar effect on protein activity.

Detection of a novel polymorphism of the CYP1A1 gene creates a number of exciting avenues for future research. A PCR assay has been developed which can detect the C to A polymorphism which causes the threonine to asparagine substitution. With this assay, 15/27 individuals from the control group were found to have the Thr-Asn^{461} variant of CYP1A1. Fourteen of these individuals were heterozygous, and had an isoleucine allele at amino acid position 462, and one individual was found to be homozygous for Thr-Asn^{461}. Confirmation of the

frequency of this novel sequence in the general population must be carried out. Conceivably this polymorphism may be race specific in a similar manner to the polymorphism of CYP1A1 found in African-Americans (Crofts *et al*, 1993). If the novel sequence is found to be present in the general population at a significant level, the effect of this polymorphism on disease susceptibility could then be investigated. Furthermore, the effect of the novel sequence on the activity of the enzyme could be studied in a similar manner to that employed by Hayashi and colleagues (1991b).

4.10.2 GSTM1 and CYP1A1 polymorphisms and disease susceptibility

While polymorphisms of GSTM1 and CYP1A1 have been shown to influence susceptibility to emphysema, these results require verification. Furthermore, the results obtained have identified associations and trends between the gene polymorphisms and susceptibility to specific patterns of emphysema, namely panacinar and centriacinar emphysema. The aetiology of these 2 patterns of emphysema is thought to be distinct (Saetta *et al*, 1994b), but the mechanism by which the aetiology might differ is unknown. This study therefore presents important evidence which may help elucidate the different mechanisms involved in the development of the patterns of emphysema. Association between the GSTM1 polymorphism and lung cancer was not identified in this study. Similarly, the CYP1A1 polymorphism was not associated with lung cancer alone, but was found to increase susceptibility to emphysema in the presence of lung cancer. Previous

reports of the association between both the CYP1A1 and GSTM1 gene polymorphisms and increased risk for lung cancer have been contradictory. Further research into the role of these enzymes in the development of lung cancer, and in particular, to the specific types of cancer, is required.

In summary, further work on the GSTM1 and CYP1A1 polymorphisms, and on other xenobiotic metabolising enzymes may provide important information which can be used to elucidate the role of the xenobiotic metabolising system of enzymes in influencing the aetiology of disease.

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Heterogeneous expression and polymorphic genotype of glutathione S-transferases in human lung

A M Cantlay, C A D Smith, W A Wallace, P-L Yap, D Lamb, D J Harrison

Abstract

***Background* – Glutathione S-transferases (GSTs) are involved in the detoxification of xenobiotics by conjugation with glutathione. One of the mu class genes of this superfamily of enzymes, GSTM1, is polymorphic because of a partial gene deletion. This results in a failure to express GSTM1 in approximately 50% of individuals. Several studies have linked GSTM1 null status to an increased risk of lung carcinoma. This study investigated the expression and distribution of GST isoenzymes in human lung, and developed a polymerase chain reaction (PCR) assay which would allow genotyping of archival, paraffin embedded lung tissue.**

***Methods* – Distribution was examined using a panel of polyclonal anti-GST antibodies for immunohistochemistry in normal tissue of 21 tumour-bearing lungs. DNA for PCR was extracted from paraffin blocks and a control group of 350 blood lysates. As a positive control each assay amplified part of GSTM4, a mu class gene which is not polymorphic but which shows strong sequence homology to GSTM1. The presence of GST in bronchoalveolar lavage fluid was sought by Western analysis.**

***Results* – Proximal airways contained pi class GST, alpha class GST, and mu class GST with expression concentrated in the brush border. In distal airspaces no alpha GST was expressed but pi GST and mu GST were present in alveolar cells and also alveolar macrophages. Pi class GST was present in bronchoalveolar lavage fluid. The PCR assay enabled genotypic determination using DNA extracted from archival material. Of the control group 56% were null at the GSTM1 locus.**

***Conclusions* – The distribution of GST isoenzymes in the lung is heterogeneous with an apparent decrease in GST in distal lung. Since GSTM1 status has already been associated with susceptibility to disease, the PCR assay developed will allow further studies of the relation between genotype and structural disorders in the lung using archival pathological material.**

(*Thorax* 1994;**49**:1010–1014)

Glutathione S-transferases (GSTs) are a superfamily of enzymes involved in the conjugation of a wide range of electrophilic substrates with glutathione, thereby facilitating detoxification and further metabolism and excretion. Regulation of GST expression is complex: it has tissue specificity in terms of distribution and it may be induced by xenobiotics, including carcinogens such as aromatic hydrocarbons and benzo(a)pyrene, which are present in cigarette smoke.[1] At least five different families exist, four cytosolic (GST P, A, M, and T) and at least one microsomal GST.[2] GSTP and M are both involved in the detoxification of 7,8-dihydroxy-9,10-oxy-7,8,9, 10-tetrahydrobenzo(a)pyrene, an active cytochrome P450-derived metabolite of benzo-(a)pyrene.[3] Although GST catalytic activity is much higher in liver, lung GST is probably of importance in the primary metabolism of many inhaled xenobiotics present in cigarette smoke or the atmosphere. Furthermore, GSTs can be induced in mouse lung by phenolic antioxidants such as butylated hydroxyanisole.[4] GSTM contains at least five distinct genes.[5] Owing to a polymorphism of human GSTM1, which comprises a deletion of part of exons 4 and 5 of the gene, about half of the population fail to express this isoenzyme.[6] Several studies have shown that individuals who fail to express GSTM1 are more susceptible to pituitary adenoma and to developing adenocarcinoma of colon and lung.[7–12] This indicates that individuals with a GSTM1 nulled genotype/phenotype have less protection against chemical stress.

In addition to xenobiotic metabolism, some human GSTs have significant selenium-independent glutathione peroxidase activity,[1] and they are also implicated in non-substrate covalent binding of some carcinogens.[13] The conjugation of leukotriene A4 with glutathione to produce the cysteinyl leukotrienes C4, D4, and E4 is a GST-dependent reaction.[14] Since leukotrienes are intimately involved in lung injury and inflammation, variation of GST expression between individuals may have significant effects on the extent of lung injury.

Several studies of GST distribution in rats[15] or mice[4] have been published but there are few reports on the distribution of GST isoenzymes in adult human lung.[16–18] In this paper we describe the distribution of GST isoenzymes in human lung tissue and bronchoalveolar lavage fluid, and describe a polymerase chain reaction assay for determining the GSTM1 genotype using DNA derived from formalin fixed archival biopsy and autopsy material.

Methods

LUNG TISSUE

Twenty one lungs or lobes of lungs obtained

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at pneumonectomy were studied. These lungs had been removed because of small peripheral tumours which proved to be lung carcinomas. For histological preparation lungs were fixed inflated with buffered formalin for 24 hours before selection of blocks from macroscopically normal lung. All but two of the patients from whom tissue was taken were known cigarette smokers. These blocks were then processed to paraffin wax and sectioned at 2 μm for immunohistochemical study.

IMMUNOHISTOCHEMISTRY

Polyclonal rabbit antisera to human GST were donated by Dr J D Hayes and have been described elsewhere.[19] Anti-GSTP was raised against purified human lung GSTP and the other antisera were raised against human liver preparations. This antibody reacted only with GSTP in liver and lung Western blots, and cross reacted with no other GST isoenzyme. Anti-GSTM was raised against liver GSTM which is the product of the GSTM1 gene. However, since GSTM4 has greater than 90% sequence homology with GSTM1,[15] it is possible that the polyclonal antibody could react with the product of both genes. The immunostaining protocol was as described previously[19] using avidin-peroxidase and 3,3-diaminobenzidine detection. The only addition was preincubation of sections with 50% pooled normal human serum in Tris-buffered saline (TBS). This preincubation resulted in significant reduction of background staining in controls using normal rabbit serum instead of polyclonal antibody. Antisera were used at a dilution of 1:200 in TBS containing 5% normal swine serum. Sections were assessed by two independent observers who then discussed the results at a twin headed microscope.

WESTERN BLOT ANALYSIS OF BRONCHOALVEOLAR LAVAGE FLUID

Four bronchoalveolar lavage fluid samples were obtained from healthy volunteers. These were not obviously blood stained. Following centrifugation to remove cellular debris, 10 ml aliquots were taken and placed in separate lengths of 14 mm Viskose dialysis tubing (Medicell International Ltd, London, UK). Protein was concentrated by dialysis against glycol methacrylate (BDH Chemicals, Poole, Dorset, UK) overnight at 4°C to give a final sample volume of 200 μl. An aliquot of this concentrate was tested for the presence of haemoglobin using proprietary Dipstix to ensure that GST detected was not simply the result of leakage from red blood cells. The protein extract was mixed with an equal volume of sample buffer (3·6 ml distilled water, 1 ml 0·5M Tris-HCl pH 6·8, 10% w/v SDS, 0·8 ml 1M dithiothreitol, 0·8 ml glycerol, and 0·05 ml 0·05% w/v bromophenol blue) and boiled for five minutes before loading on a 7·5% polyacrylamide gel. Purified GSTP (Sigma, UK) and liver cytosol were included as controls for antibody specificity. Protein was transferred to nitrocellulose and probed with GSTP antibody. Detection was by enhanced chemiluminescence (Amersham, UK).

POLYMERASE CHAIN REACTION (PCR)

Preparation of DNA

DNA was extracted from paraffin blocks, mounted tissue sections and peripheral blood using a modification of published techniques.[20,21] Sections were cut at 10 μm from blocks or scraped from slides and placed in an Eppendorf tube. To this was added 400 μl buffer A (50 mM KCl, 2·5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8·0), 0·45% Nonidet P-40, 0·45% Tween-20) containing 200 mg/ml proteinase K. The sections were then incubated for two hours at 55°C before boiling for 20 minutes and storing at 4°C. In PCR reactions 15 μl of this crude solution was used as template DNA. Control blood samples were collected from a randomly selected, anonymous white population (n = 350) at a routine blood donor clinic.

PCR assay

The PCR buffer was a standard mix of nucleotides and contained 3% DMSO. The amplification was achieved by adding five units of Taq polymerase (Promega, UK) in a hot start, and undergoing 35 amplifications of 59°C for 30 seconds, 72°C for 90 seconds, and 94°C for 30 seconds. Amplified DNA was electrophoresed in 3% agarose gels. DNA was visualised by ethidium bromide staining and examined under ultraviolet irradiation.

Primers

The primer strategy used a modification of that used by Zhong *et al*[9] and Shea *et al*[22] (fig 1). This method allowed the amplification of both GSTM1 and GSTM4 and hence provided a positive control for each reaction since GSTM4, which is not polymorphic,[5] was always amplified. This was particularly important as DNA extracted from archival paraffin blocks may be of poor yield. A loss of GSTM1 identified in this way could therefore be distinguished from a failed PCR reaction.

The following oligonucleotide primers were used in the PCR reaction:

22y:
5′-CTGCCCTACTTGATTGATGG-3′
23y:
5′-ATCTTCTCCTCTTCTGTCTC-3′
24y:
5′-TTCTGGATTGTAGCAGATCA-3′

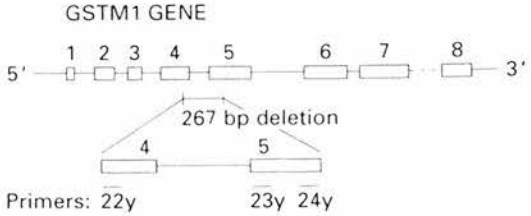


Figure 1 Schematic structure of the GSTM1 gene. The upstream primer (22y) is situated in exon 4, whereas both downstream primers (23y and 24y) are located in exon 5. Both 22y and 23y anneal to GSTM4 in addition to GSTM1 and thus serve as a positive control for the PCR assay. Primer 24y is specific to GSTM1.

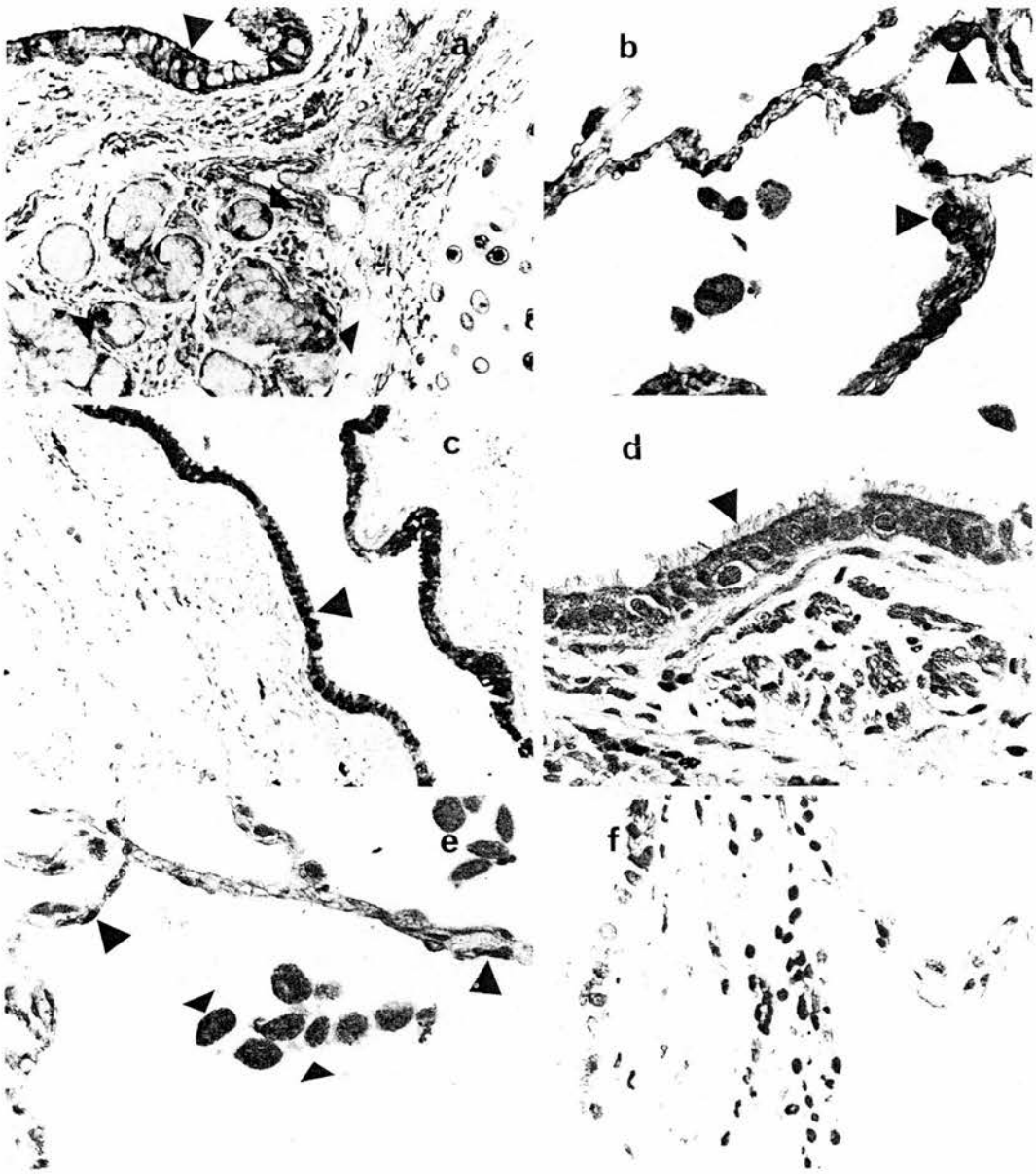


Figure 2 (a) GSTP in bronchial epithelial cells (big arrowhead), serous cells (small arrowhead), and chondrocytes (bottom right side of photomicrograph). (b) Alveolar macrophages in the lumen and lining cells, particularly type II alveolar cells (arrowheads), stained strongly for GSTP. (c) GSTA presence limited to airway epithelium (arrowhead); alveoli and macrophages not stained. (d) GSTM present in bronchial epithelium (arrowhead) including the brush border. (e) Heterogeneous staining of alveolar macrophages for GSTM (small arrowhead) and weak staining of alveoli (big arrowhead) which was more intense than a negative control sample. (f) Negative control showing no significant reaction product. All that is visible is nuclei counterstained with haematoxylin. There is no immunoreactivity.

The primers 22y and 23y, when used together in a PCR reaction, amplify a DNA fragment of 202 bp in length, while the use of 22y and 24y together results in the amplification of a fragment 275 bp long.

Results

LOCALISATION OF GST ISOENZYMES IN LUNG TISSUE

There was complete agreement between the two observers on the pattern of distribution of GSTs in the lung samples. No formal attempt was made to quantify the amount of GST present, but cases were scored as positive when the staining intensity was greater than a control slide for which non-immune rabbit serum was substituted for primary antiserum (fig 2f).

GSTP

GSTP was present in every lung examined. Bronchial epithelial cells were strongly positive with reactivity noted in cytoplasm, brush border, and most nuclei. Muscle, nerve, serous glands, and chondrocytes were also positive (fig 2a). Terminal bronchioles and both type 1 and type 2 pneumocytes contained GSTP. Endothelial cells were not stained. Alveolar macrophages were variably positive (fig 2b). No differences were noted between smokers and the two tissue samples from non-smokers.

GSTA

There was GSTA present in the cytoplasm, some nuclei, and brush border of most, but not all, bronchial epithelial cells. Some chondrocytes stained but alveolar lining cells

and macrophages were consistently negative (fig 2c).

GSTM

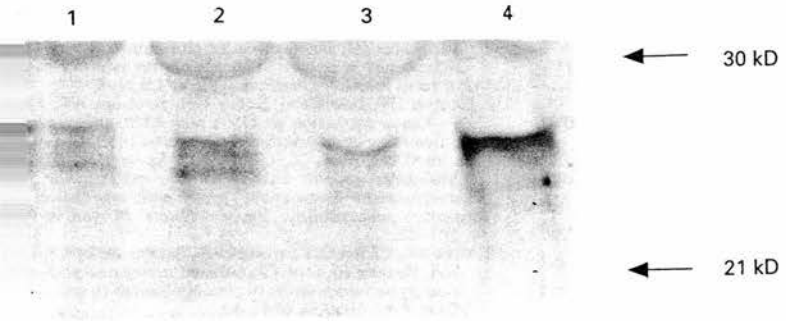
The staining intensity for GSTM was less than for other isoenzymes. Only two cases of the 21 studied showed strong staining for GSTM, seven were weakly stained, and the remainder were negative. The pattern of distribution was the same as for GSTP, however – that is, bronchial epithelium, types 1 and 2 alveolar cells, and macrophages (figs 2d and 2e).

LOCALISATION OF GST ISOENZYMES IN BRONCHOALVEOLAR LAVAGE FLUID

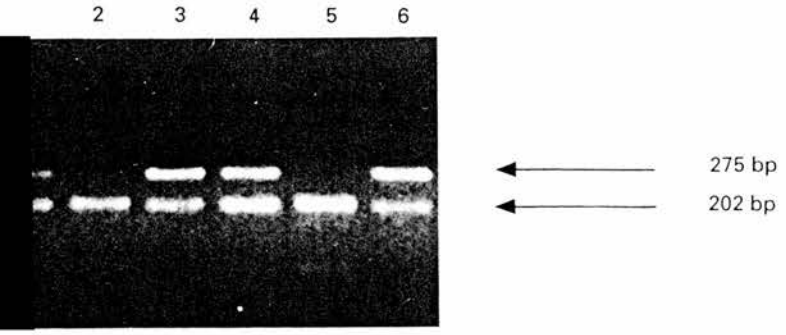
All four samples showed reactivity with GSTP antibody, although in two cases GSTP appeared to be partly degraded (fig 3). In three cases no evidence was found to indicate that haem was present. In one sample there was trace positivity by testing with Dipstix.

POLYMERASE CHAIN REACTION ANALYSIS

PCR analysis resulted in specific fragments which were easily interpreted when visualised on agarose gels as described (fig 4). Of 16 DNA samples extracted from archival lung tissue nine were null at the GSTM1 locus. There was no correlation demonstrable between GSTM1 genotype and immunophenotype. Analysis of 350 random DNA samples extracted from blood samples showed that 198 individuals (56%) were null for GSTM1.



re 3 Western blot analysis of bronchoalveolar lavage fluid probed with antibody nst GSTP. In lanes 1 and 2 lower molecular weight bands reacting with antibody can en, consistent with degradation of protein. This pattern is not seen with the positive ol sample of purified GSTP.



e 4 Examination of DNA amplified by polymerase chain reaction from blood s and paraffin embedded tissue clearly identified the null GSTM1 homozygous ype (lanes 2 and 5) as opposed to the presence of the intact wild type allele (lanes 1, nd 6). In each lane the constant lower molecular weight fragment represents control fication of GSTM4.

Discussion

Our findings suggest that GST mediated protection against xenobiotic and oxidative stress in the lung can be divided into two functional compartments. In proximal airways all three cytosolic forms (GST P, A, and M) were present in lining epithelium, whereas the distal alveolar compartment contained GSTP and GSTM but no GSTA in both type 1 and 2 pneumocytes as well as macrophages. This is consistent with biochemical studies which showed that GSTP is the predominant lung isoenzyme.[23,24] In a previous study of GST in human lung development all isoenzymes were identified, with GSTP being the predominant isoenzyme. The levels of GSTP were higher during fetal development, with strong reactivity in bronchi and protoalveoli, and fell during late gestation.[25] In another study of only three human lungs[16] GSTP, GSTA, and GSTM were all reportedly present in alveoli. This is inconsistent with the work of Fryer and colleagues in fetal lung[17] and with our own study. The discrepancy may be because an immunofluorescence assay was used in the study of Awasthi *et al*[16] which is more sensitive but also produces higher backgrounds. When we omitted the preincubation of sections with a blocking buffer containing 50% human serum we also obtained diffuse alveolar staining with all antibodies. Our findings are in broad agreement with the distribution of GST enzymes reported by Antilla and colleagues.[18]

In previous studies of localisation of GST in mouse and rat lungs[4,15] cellular GST was restricted to bronchial epithelial cells, including the brush border, with virtually no GST identifiable in alveoli. In addition, GSTP in rat lung was also identified as an extracellular protein in association with elastin.[15] This was apparent in electron but not light microscopic preparations. We found no evidence for extracellular, interstitial GST but we have suggested that GSTP is present in bronchoalveolar lavage fluid. Another possibility for the presence of GSTP in bronchoalveolar lavage fluid is by leakage from red blood cells. However, three of the samples were negative for haem when tested and the fourth showed a trace of haem. This may indicate the presence of blood contamination, but another possibility is that haem present in cytochromes P450 was detected. Cytochromes P450 are thought to be a normal constituent of the epithelial lining fluid in the lung (R Richards, personal communication). Furthermore, our findings are consistent with a previous study in which both GSTA and GSTP were found in bronchoalveolar lavage fluid of tumour-bearing lungs.[26]

Cigarette smoke contains a plethora of chemicals, many of which are electrophilic or become electrophiles after metabolism principally by cytochrome P450 enzymes. These compounds serve as substrates for GSTP, GSTA, and GSTM. Proximal airways contained GSTA

which is involved in selenium-independent glutathione peroxidation reactions, and which may detoxify DNA and lipid hydroperoxides.[1] Its presence in the brush border is therefore suggestive of a role for GSTA in the protection of bronchial epithelium. Most acute damage following cigarette smoke inhalation is seen in small distal airways and alveoli,[27] a site lacking this selenium-independent peroxidase protection. In individuals null at the GSTM1 locus it therefore follows that the level of protection in distal airways afforded by GSTs is significantly reduced. Several reports have suggested that the expression of GSTM in lung shows evidence of polymorphism which is consistent with the gene expressed being GSTM1 rather than GSTM4.[17 23] In one study of 10 lungs, however, no evidence of polymorphic expression was seen although the level of GSTM catalytic activity was markedly variable between cases.[24] In this present study we cannot confirm that the immunohistochemical expression seen was specifically the polymorphic enzyme GSTM1 rather than the non-polymorphic GSTM4. Further studies are in progress to address this problem by analysis of mRNA.

Support for the association of GSTM1 status and disease susceptibility comes from in vitro studies of leucocytes which showed that cells from GSTM1 null individuals were more prone to epoxide-induced DNA injury and sister chromatid exchange.[28] For this reason the determination of GSTM1 genotype is important in considering individual susceptibility to xenobiotics by inhalation of cigarette smoke. This is equally relevant to metabolism occurring in the lung, as well as other organs including the liver where there is high expression of the enzyme.[29]

The presence of GSTP in alveolar macrophages and bronchoalveolar lavage fluid is interesting. GSTP functions as a homodimer and it can be readily inactivated by oxidation of reduced sulphydryl groups.[13] It may therefore function as a "sacrificial reactive protein" in addition to being a GST-dependent enzyme. Indirect support for this suggestion is provided from a study where cigarette smoke reduced GSTP activity in alveolar macrophages,[29] despite the fact that cigarette smoke contains many chemicals which might increase GSTP expression. In these cells GSTP activity may have been reduced by oxidative degradation rather than by reduced expression of the protein.

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Association between the CYP1A1 gene polymorphism and susceptibility to emphysema and lung cancer

A M Cantlay, D Lamb, M Gillooly, J Norrman, D Morrison, C A D Smith, D J Harrison

Abstract

***Aim*—To investigate cytochrome P4501A1 (CYP1A1) polymorphism and susceptibility to emphysema and lung cancer.**

***Methods*—A novel polymerase chain reaction (PCR) for genotyping the CYP1A1 polymorphism, corresponding to putative low or high enzyme activity, was developed to genotype lung cancer resection samples which had been assessed macroscopically for the presence of centriacinar and panacinar emphysema. Samples were collected and genotyped from a group of patients with chronic obstructive airways disease. A control group of anonymous blood donations was genotyped to determine the basal levels of the polymorphism in the Scottish population.**

***Results*—The high activity allele of the CYP1A1 gene is associated with susceptibility to centriacinar emphysema and lung cancer but not panacinar emphysema. CYP1A1 polymorphism is not linked to lung cancer in the absence of emphysema, nor to chronic obstructive airways disease which is the clinical manifestation of emphysema, particularly of the panacinar type.**

***Conclusions*—Susceptibility to emphysema and lung cancer is associated with polymorphism of the P4501A1 gene. A trend towards damage of centriacinar pattern has been detected, which supports the theory that centriacinar emphysema results from local, direct damage to the respiratory bronchioles from exposure to cigarette smoke.**

(*J Clin Pathol: Mol Pathol* 1995;**48**:M210–M214)

Keywords: CYP1A1 polymorphism, emphysema, lung cancer, cigarette smoke.

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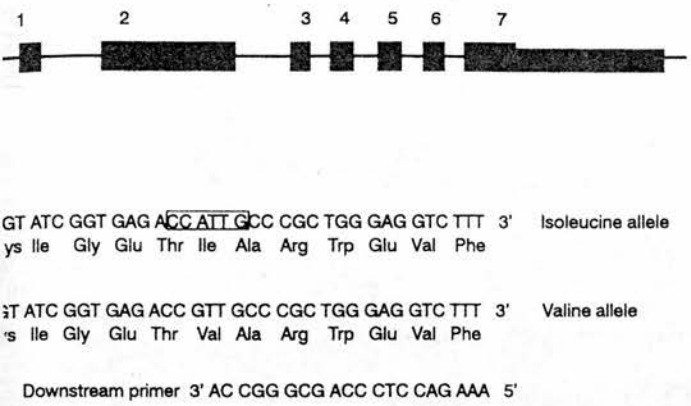
A single inhalation of cigarette smoke contains approximately 10^{16} reactive species,[1] which are both cytotoxic and genotoxic. Lung tissue damage occurs through reactive species' destruction of cell membranes and structural components.[2] Procarcinogens and oxidants cause DNA mutations,[3] which may lead to cancer initiation.[4] Cigarette smoke can stimulate macrophages to release chemotactic factors which recruit inflammatory cells[5 6] and nicotine has been shown to be chemotactic.[7] These cells release proteases, particularly elastase, during their inflammatory response.[8] In rats emphysema has been induced by administration of elastase to the lungs.[9] Antiproteases protect the structure of the lung from destruction by proteases such as elastase and collagenase.[8] Oxidants present in cigarette smoke inhibit the action of antiproteases, further reducing the protective capacity of the lungs.[10] Cigarette smoke causes at least two diseases of major clinical importance, lung cancer and emphysema.

The lungs are protected from the toxic effects of cigarette smoke by antiproteases, antioxidants and xenobiotic metabolising enzymes.[1] In the lung both cancer and emphysema induced by cigarette smoke may result from variation in the protective capacity of lung tissue. Xenobiotic metabolising enzymes metabolise exogenous compounds, which may be toxic, to forms which are more easily excreted in the urine or bile.[11] This metabolism may be an important primary defense against lung injury resulting from exposure to cigarette smoke. Variation in interindividual expression of metabolising enzymes may result in the differential ability of tissues to protect against disease.

Several of the major xenobiotic metabolising enzymes are polymorphic at the genetic level. These include cytochrome P4502D6,[12] glutathione S-transferase M1[13] and N-acetyl transferase.[14] Association between some of these enzyme polymorphisms and cancer susceptibility has been demonstrated[15]—for example, both cytochrome P4502D6 and glutathione S-transferase M1 polymorphisms have been associated with susceptibility to lung cancer, and the N-acetyl transferase 2 gene polymorphism has been implicated in bladder and colon cancers.

Cytochrome P4501A1 is a phase I metabolising enzyme which may activate procarcinogens and xenobiotics to their full carcinogenic and electrophilic forms.[16] Expression of this enzyme is primarily extrahepatic[17] and is widespread in the lung.[18] CYP1A1 expression is inducible by polycyclic aromatic hydrocarbons such as benzo[a]pyrene and 3-methylcholanthrene,[19 20] which form a major component of cigarette smoke.[21] A point mutation in exon 7 of the CYP1A1 gene results in an amino acid substitution from an isoleucine to a valine.[22] This mutation occurs in the region of the gene which encodes the heme binding motif of the protein, and studies of benzo[a]pyrene metabolism have shown that the valine protein demonstrates almost twice the enzyme activity of the isoleucine protein.[16] This polymorphism is linked to a *MspI* restriction enzyme fragment length polymorphism in the 3′ region of the gene,[22] which is associated



hematic diagram of the CYP1A1 gene. Sequences of the two alleles of the ie are shown, along with the downstream primer, which introduces a base amplimer. Following PCR, the base mismatch introduced into the amplimer oI site (box) in the Isoleucine allele only, the site is absent in the Valine

with susceptibility to lung cancer in studies of Japanese populations.[23 24] Studies of white populations have been unable to demonstrate similar associations, which may be a reflection of the lower prevalence of these polymorphisms in whites.[25 26] Much larger study groups are required to investigate the polymorphism in the latter to generate sufficient numbers of rare alleles to permit statistical analysis of disease association.

To investigate involvement of the cytochrome P4501A1 gene polymorphism in susceptibility to lung disease, we collected lung samples which were resected for carcinoma. We also obtained blood samples from patients with chronic obstructive airways disease. Chronic obstructive airways disease is believed to be the clinical manifestation of emphysema[27 28]; however, this clinical manifestation is more commonly seen in panacinar rather than centriacinar emphysema.[29] Previous strategies for analysis of the exon 7 CYP1A1 polymorphism have made use of specific differential polymerase chain reaction (PCR) priming of the allelic variants of the gene, and have relied on high specificity of priming.[30] We developed a novel PCR assay to genotype paraffin wax embedded lung tissues for the exon 7 polymorphism of the CYP1A1 gene, as use of high specificity primers relies on very stringent conditions which may reduce the yield of PCR amplimers.

Methods

Lung resection specimens (n = 129) from smokers with lung cancer were collected. The presence, type and extent of emphysema was assessed macroscopically in non-involved lobes and this assessment was confirmed by morphometric microscopic analysis of alveolar wall surface area per unit volume (AWUV).[31] All emphysema cases were of mild or moderate disease severity, with a forced expiratory volume in one second (FEV_1) of at least 1·4, as patients must be sufficiently fit to recover from surgery.

Blood samples collected from a blood donor clinic served as an anonymous control population of 281 individuals. Fifty one blood samples from patients with clinical lung disease were also collected.

DNA EXTRACTION AND PCR ANALYSIS

DNA extraction from paraffin wax embedded lung samples with no evidence of carcinoma and blood samples was carried out as described previously.[32] Genotyping was carried out by PCR analysis using buffer and 1·5 mM $MgCl_2$ (Promega, Southampton, UK), 150 mM deoxynucleotides (Pharmacia, Milton Keynes, UK), 5% DMSO (Sigma, St Louis, Missouri, USA), 25 pmoles primer (Oswell DNA Services, Edinburgh, UK), and 2·5 units of Taq polymerase (Promega, UK). The primers used were: upstream, 5′-AAAGGCTGGG-TCCACCCTCT-3′; and downstream, 5′-AAAGACCTCCCAGCGGGCCA-3′ (fig 1).

The downstream primer incorporated a mismatched base to engineer a *Nco*I restriction enzyme site in the PCR products derived from the Ile^{462} allele of the gene. This restriction site is lost in the Val^{462} allele of the gene. The primers amplify both alleles and the genotypes are distinguished by *Nco*I digestion of the products. A *Nco*I restriction enzyme site located upstream of the mutation in either genotype serves as a positive control for PCR product digestion. PCR products were electrophoresed in 3% NuSeive and SeaKem agarose (FMC Bioproducts, Rockland, Maine, USA), and restriction enzyme digestion fragments were electrophoresed in 3% Metaphor agarose (FMC Bioproducts).

STATISTICAL ANALYSES

Odds ratios and confidence intervals were used to analyse the frequencies of the CYP1A1 genotypes, significance testing was by χ^2 analysis.[33]

Results

DISEASE INFORMATION

No significant differences in age, sex, or tumour type were found within the study populations. The median FEV_1 of chronic obstructive airways disease cases was 1·1, and 2·2 for the biopsy study group. Smoking histories were recorded where possible in pack years of exposure, and the median pack years for the chronic obstructive airways disease group was 35, while the biopsy cases had a median of 46 pack years.

Of the 129 lung cancer samples studied, 42 had no macroscopic emphysema, 34 showed centriacinar patterns of damage, 17 samples had panacinar emphysema, and 36 lung biopsy specimens had both centriacinar and panacinar forms of emphysema.

GENOTYPING OF CYP1A1 GENE POLYMORPHISM

Each amplimer analysed had a diagnostic *Nco*I restriction enzyme site and a second constant control *Nco*I site, which enabled distinction of the CYP1A1 genotype of the individual. PCR analysis and subsequent enzyme restriction

Table 1 CYP1A1 genotypes for the disease groups and control group for statistical comparison

Subjects	Ile/Ile genotype	Ile/Val genotype	Val/Val genotype	Total cases	χ^2 v controls	p value	Odds ratio	95% confidence limits
Controls	245 (87%)	33 (12%)	3 (1%)	281	—	—	—	—
All lung cancers	106 (82%)	21 (16%)	2 (2%)	129	1·81	Not significant	1·48	0·83–2·61
Cancer with no emphysema	39 (93%)	3 (7%)	0	42	1·11	Not significant	0·52	0·16–1·77
Cancer with emphysema	67 (77%)	18 (21%)	2 (2%)	87	5·33	0·021	2·03	1·10–3·73
Chronic obstructive airways disease	42 (82%)	9 (18%)	0	51	1·29	Not significant	1·46	0·66–3·25

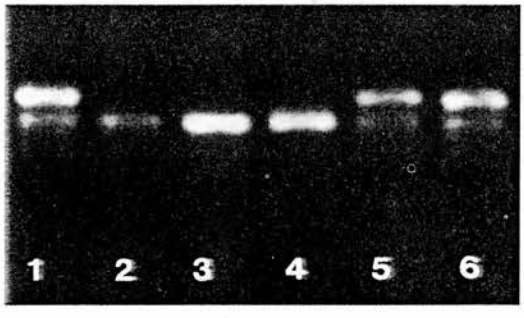
Table 2 CYP1A1 genotypes of cases with emphysema and lung cancer divided by pattern of emphysema

Types of emphysema	Ile/Ile genotype	Ile/Val genotype	Val/Val genotype	Total cases	χ^2 v controls	p value	Odds ratio	95% confidence limits
Centriacinar alone	25 (73·5%)	9 (23·5%)	0	34	4·62	0·035	2·45	1·06–5·67
Panacinar alone	15 (88%)	1 (6%)	1 (6%)	17	0·02	Not significant	0·91	0·20–4·10
Both	27 (75%)	8 (22%)	1 (3%)	36	3·89	0·049	2·25	0·98–5·17

analysis yielded specific fragments which were easily visualised on agarose gels (fig 2).

The control group numbered 281 samples, of which 87% (244/281) were homozygous for the Ile^{462} allele, 12% (34/281) were heterozygous and 1% (three of 281) were homozygous for the valine allele of the CYP1A1 gene (table 1). In comparison, of the 129 lung resection cases, 82% (105/129) exhibited homozygosity at the Ile^{462} allele, 16% (21/129) were heterozygous for Ile^{462} while 2% (three of 129) exhibited Val^{462} homozygosity. This group was not significantly different from the control population.

(A)



(B)

Figure 2 (A) Results of a PCR of the CYP1A1 gene. DNA molecular marker V is shown in the lefthand lane, with PCR amplimers in the remaining lanes. The amplimer (322 base pairs) lies between fragments of 434 and 267 base pairs of the marker. (B) Results of a NcoI *digestion of the CYP1A1 amplimer. The two bands represent the two alleles of the gene, the higher molecular weight fragment (250 base pairs) representing the uncut valine allele of the gene, and the lower molecular weight band (231 base pairs) representing the cut isoleucine allele of CYP1A1. Lanes 1, 5 and 6 therefore represent heterozygotes for the two alleles, while lanes 2, 3 and 4 contain digestions from individuals homozygous for the common Isoleucine allele.*

In the group of patients with chronic obstructive airways disease, of the 51 samples, 82% (42/51) were homozygous for Ile^{462} and 18% (nine of 51) were heterozygous for the polymorphism. No individuals homozygous for the valine allele were found in this disease group, and no significant difference between this study group and the controls was seen.

To investigate the CYP1A1 gene polymorphism and susceptibility to emphysema, the total lung cancer study group was divided according to the pattern of emphysematous damage in the lung (table 2). When this was done, 42 samples showed no evidence of emphysema and of these, 93% (39/42) were Ile^{462} homozygotes; 7% (three of 42) were heterozygotes. Centriacinar emphysema only was found in 34 cases and of these, 73·5% (25/34) were homozygous for the valine allele and 26·5% (nine of 34) were Ile/Val. Panacinar emphysema alone was present in 17 samples and 88% (15/17) were Ile^{462} homozygotes, 6% (one of 17) were Ile/Val, and 6% (one of 17) were homozygous for the Val^{462} allele. Both panacinar and centriacinar emphysemas were found in 36 of the lung cancer samples and homozygotes for the isoleucine allele accounted for 75% (27/36) of these cases, 22% (eight of 36) were heterozygotes and 3% (one of 36) were Val^{462} homozygotes.

Of these disease groups, only those patients with centriacinar emphysema alone and both centriacinar and panacinar emphysema differed significantly from the controls. Odds ratios were 2·45 (95% confidence limits 1·06–5·67) and 2·25 (95% confidence limits 0·98–5·17), respectively.

Discussion

We have found a statistically significant association of the rare, putative high activity allele

of CYP1A1 in cases with both lung cancer and emphysema (odds ratios of 2·45 and 2·25) but not in cases with chronic obstructive airways disease or cancer alone. Clinical cases of chronic obstructive airways disease do not represent just emphysema but also include chronic abstructive airways disease secondary to chronic bronchitis. This may explain why a lack of CYP1A1 association is seen for this condition, but association with the polymorphism is seen with pathological emphysema. Previous studies investigating CYP1A1 and lung cancer have provided conflicting data. Whereas in Japanese populations CYP1A1 conferred a threefold increased risk of lung cancer, similar studies of Scandinavian populations have failed to find any association.[25 26] No study to date has considered the possibility that CYP1A1 may confer susceptibility not just to lung cancer but also to other forms of lung disease, such as emphysema. What do the results we have obtained suggest?

Firstly, although our data indicate that CYP1A1 is associated with lung cancer and emphysema, the increased relative risk we have found is rather small and is only significant when both diseases are present. However, emphysema and lung cancer are extremely prevalent diseases, hence even a slight increase in disease susceptibility conferred by this polymorphism may account for a large proportion of cases.

Secondly, the increased susceptibility to lung disease conferred by the CYP1A1 polymorphism appears to be an early event, occurring at a stage before the injury response pathway leading to cell death and inflammation has diverged from that leading to mutagenesis and eventual tumour progression. This is in keeping with the likely position of CYP1A1 in the pathway of cigarette smoke metabolism—that is, it is proximate to the insult by nature of its expression in lung parenchyma and role in phase I metabolism.

Thirdly, the effect of CYP1A1 genotype and phenotype on disease susceptibility will be influenced by other mechanisms of disease development. These may be more important than CYP1A1 in determining the likelihood of cigarette smoke injury to result in predominantly genotoxic or cytotoxic injury. Thus, certain protective mechanisms such as the glutathione dependant system, epoxide hydrolase, differences in proteolytic enzymes, or antiproteases may reduce the likelihood of cell damage caused by lipid or protein peroxidation leading to emphysema. It is perhaps significant that the cases with both lung cancer and emphysema had mild or moderate emphysema whereas the clinical group with chronic obstructive airways disease had severe disease—that is, CYP1A1 polymorphism contributes little towards susceptibility to severe, non-neoplastic lung injury.

Conversely, differences in DNA repair and other genes involved in recognising and eliminating DNA damage may be very important in determining whether or not tumorigenesis can occur. These mechanisms are likely to differ between individuals and consistent with this is the presence of known polymorphisms/mutations—for example, in the p53 gene and in the DNA mismatch repair system.[34–36] These polymorphisms or mutations may contribute to interindividual differences in the capacity to eliminate DNA damage and therefore prevent mutations leading to tumorigenesis.

Only in cases with no bias towards, or away from, non-neoplastic or neoplastic lung disease would CYP1A1 be expected to have a detectable effect on the susceptibility to disease rather than simply a more subtle underlying modification of mechanisms and responses to injury.

The results that we have obtained in this study show that a genetically determined difference in xenobiotic metabolism, in this case components of cigarette smoke, may alter the response of cells or tissue to injury and thus susceptibility to disease.

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